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The Response of Unpolarized Macrophages (RAW 264.7)/Keratinocytes (PAM-212) Monolayer and Co-Culture System to Herpes Simplex Virus Type 1 (HSV-1) Replication During the Infection.

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The Response of Unpolarized Macrophages (RAW 264.7)/Keratinocytes (PAM-212) Monolayer and Co-Culture System to Herpes Simplex Virus Type 1 (HSV-1) Replication during the Infection.

A thesis submitted in partial fulfillment

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Master of Science.

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY FAHAD MOHAMMED ALRADI ENTITLED The Response of Unpolarized Macrophage (RAW 264.7)/Keratinocytes (PAM-212) Monolayer and Co-Culture System to Herpes Simplex Virus Type 1 (HSV-1) Replication during the Infection. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Alradi, Fahad Mohammed. M.S. Department of Microbiology and Immunology, Wright State University, 2018. The Response of Unpolarized Macrophage (RAW 264.7)/Keratinocytes (PAM-212) Monolayer and Co-Culture System to Herpes Simplex Virus Type 1 (HSV-1) Replication during the Infection.

Keratinocytes and neurons cells are the main target for Herpes Simplex Virus Type 1 (HSV-1) invasion. Moreover, keratinocytes are the most abundant cell types in the epidermis layer in the skin. Therefore, they are the first cells to encounter HSV-1 in the primary infection. Next, the virus reaches the nerve endings and is transferred to neuronal cells as a result from the primary infection. In between these two events, innate immune cells including monocytes and macrophages response is activated and recruited to the infection site. In this study, keratinocytes (PAM-212) and murine macrophage (RAW 264.7) cell lines were utilized to investigate the response of macrophages (RAW 264.7) and keratinocytes (PAM-212) to HSV-1 infection and propagation in vitro. In this study, initially keratinocytes (PAM-212) and macrophages (RAW 264.7) and were studied either infected or uninfected with HSV-1 at MOI 0.1 in a monolayer model. Cell lines were co-cultured at ratio 1:5 (macrophages : keratinocytes) respectively in the co-culture model [1]. The results showed some differences and similarities in cell viabilities, and viral replication between keratinocytes (PAM-212) and macrophages (RAW 264.7) at 24, 48, and 72 hours. In both the monolayer and the co-culture model, cells were exhibited morphological changes including irregularly shaped or rounded cells, enlargement of cell size, and cells appeared in different density compared to neighboring cells especially in the earlier phase of infection at 24 and 48 hours.

In contrast, at 72 hours cells were degraded, detached, and more debris was observed in the medium. In like manner, our results of cell viability showed some variations after 24, 48, and 72 hours which confirms the HSV-1 infection. Furthermore, plaque assay showed a significant decrease in HSV-1 titers at 24 hours in the co-culture model compared to keratinocytes and macrophages monolayers. However, the keratinocyte monolayer appeared to tolerate HSV-1 replication and virus titers kept increasing in all time-points.

On the other hand, the macrophage monolayer exhibited a noticeable decrease in the virus concentration at 48 hours, indicating the role of macrophages restricting viral replication. The effect of macrophages was diminished as time increased, but still exhibited a reduction in the virus titers compared to the keratinocytes and co-culture at 72 hours. That suggests a pivotal role of macrophages to induce immune response that limits viral replication as we proposed in our hypothesis. We still believe in the role of the cytokines produced by macrophages such as interferons IFN-α/β and interleukin IL-1α/β as antiviral therapy.

HYPOTHESIS

The hypothesis of this study is that mimicking an in-vivo immune response can be induced in a co-culture model as macrophages (RAW 264.7) exposed to keratinocytes infected with HSV-1. Macrophages (RAW 264.7) would restrict HSV-1 replication within keratinocytes and clear the infection.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

HSV-1 = Herpes Simplex Virus Type 1

M0 = Unpolarized macrophages phenotype

PAM-212 = Keratinocytes cell line

HVEM = Herpes Virus Entry Mediator

3-O-HS = 3-O-sulfotransferases

HS = Heparan Sulfate

PILRα = Paired Immunoglobulin-like type 2 Receptor alpha

TLR = Toll-like Receptor

MyD88 = Myeloid differentiation primary response 88

PRRs = Pattern Recognition Receptor

IL-1R = Interleukin-1 Receptor

MHC I & II = The major histocompatibility complex class 1 & 2

rpm = Revolutions per minute

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INTRODUCTION

Herpes Simplex Virus type 1(HSV-1), is one of the most prevalent human pathogens among Herpesviridae family and causes oral and genital herpes infection, ocular infection, and encephalitis [2]. It infects numerous types of cells, including keratinocytes and neurons, which are the main target for a HSV infection. However, the HSV-1 infection may turn systemic and fatal in young children and immunocompromised people [3]. Moreover, HSV-1 is contributed to 60 – 80% of herpesvirus infections in human worldwide and persists for life-long in people who are exposed to the infection [4]. The HSV-1infection may also induce some symptoms represented in fever, painful vesicles, tingling, abnormal sensation, itching, and burning in the lesions occurring in mouth and face [5]. HSV-1 consists of a linear genome, double-stranded DNA, and envelope that carries approximately 13 glycoproteins on the lipid envelope involving in various functions leading to integration into the host cell [6] (Figure: 1).

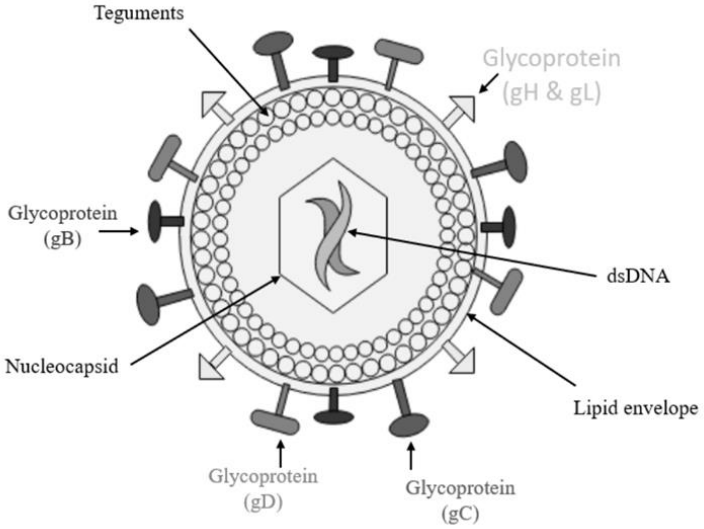


Figure 1: The Structure of Herpes Simplex Virus Type 1 (HSV-1).

Keratinocytes and HSV-1 Entry:

Skin is a very important natural defense barrier to protect the body from external environments [7]. It is composed of two main layers: dermis and epidermis. The dermis layer is rich in nerves and blood vessels while the epidermis layer consists of different cell types including melanocytes, Langerhans cells, and Merkel cells, but keratinocytes are the major resident in the epidermis in the skin [8]. Oral mucosa has a similar structure to the skin and rich in keratinocytes [9]. More importantly, it has been found that keratinocytes can produce cytokines, that drives immune response, as well as many other growth factors which are all essential for maintaining the natural homeostasis [10]. What we mean to say is that keratinocytes have a pivotal role playing as a physical barrier and immune response inducer against invaders.

When Herpes Simplex Virus Type 1(HSV-1) encounters the host cell, it may utilize both endocytosis and fusion within the plasma membrane pathways by binding to the HveC gD receptor which is also known as nectin-1; suppression of this receptor leads to reduction of the HSV-1 entry on murine keratinocytes [11]. The importance of the nectin-1 receptor that mediates the HSV-1 entry has been reported in many studies. The glycoprotein D (gD) facilitates the HSV-1 entry by interacting with the nectin-1 receptor on the host cells [12]. In addition to the nectin-1 receptor, there are many receptors involving virus entry into a host cell, including herpesvirus entry mediator (HVEM) and 3-O-sulfated heparan sulfate (HS) which interact with glycoproteins gB, gH, and gL [6].

The Role of Macrophages During HSV-1 Infection:

Macrophages are derived from monocytes, and they play important roles in regulating the innate immune response during inflammation and infection. Hence, macrophages present and emigrate within the bloodstream, and some of them reside in tissues [13]. During infection, macrophages play critical roles in homeostasis, tissue repair, and inducing immune response. Thus, macrophages are found almost all over the body, and they act as phagocytic cells which engulf and kill invaders and foreign substances [14]. While in the HSV-1infection, the virus binds to macrophage utilizing the toll-like receptor (TLR) and activates the myeloid differentiation primary response 88 (MyD88) signaling the pathway leading to the production of pro-inflammatory cytokines such as anti-viral interferon; in addition, macrophages can eliminate the HSV-1 infection in various ways that includes the production of reactive oxygen species (ROS) and nitric oxide (NO) [15].

In this study, the PAM-212 murine keratinocytes and RAW 264.7 macrophage cell line were selected for studying the role of macrophages in restricting HSV- 1 replication during infections. The hypothesis of this study was that mimicking the in-vivo immune response will be induced by macrophage when keratinocytes is infected with HSV-1. The effects of HSV-1 on cell viability, morphology, and replication at 24, 48, and 72 hours were observed in each cell types and in co-culture. The RAW 264.7 macrophage cell line would restrict HSV-1 replication within keratinocytes and clear the infection.

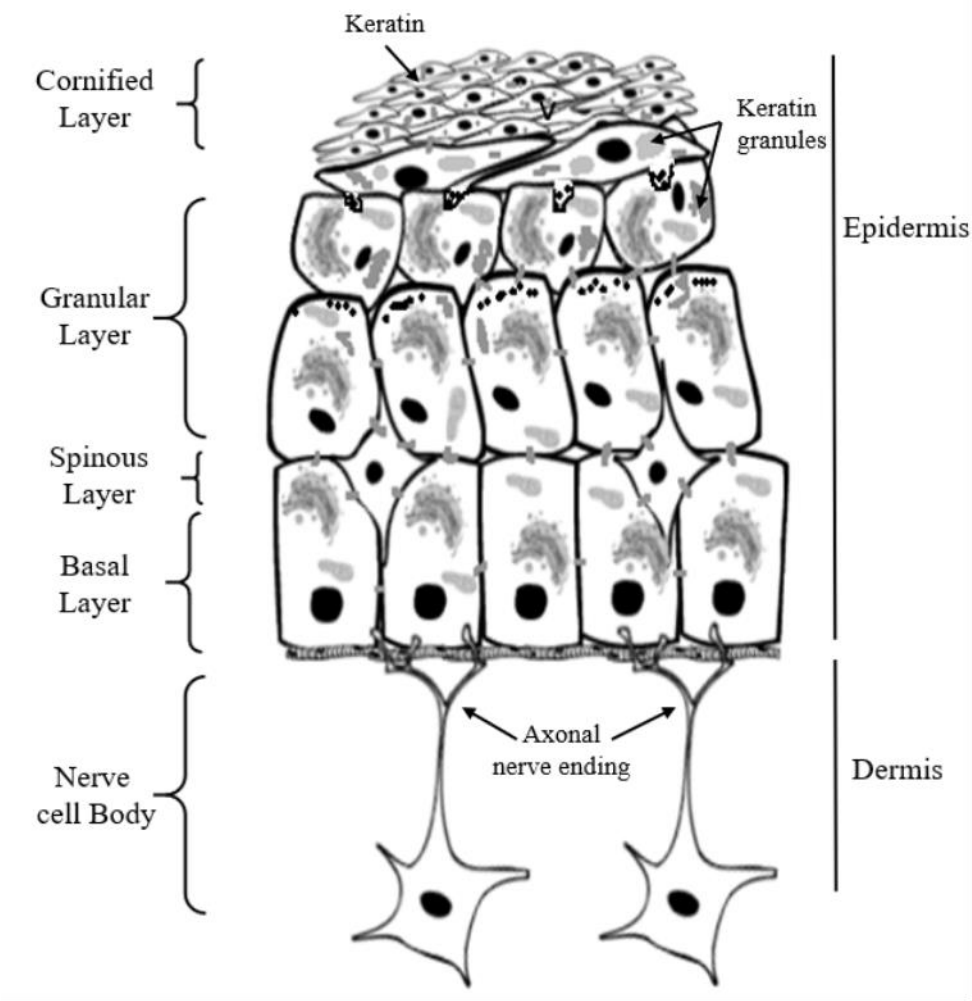


Figure 2: This Diagram Illustrates the Main Layers of the Skin. The epidermis layer is the first layer in the skin and it is composed of several layers and different types of cells, mainly keratinocytes. The dermis layer is the next one to the bottom and it consists nerves and blood vessels.

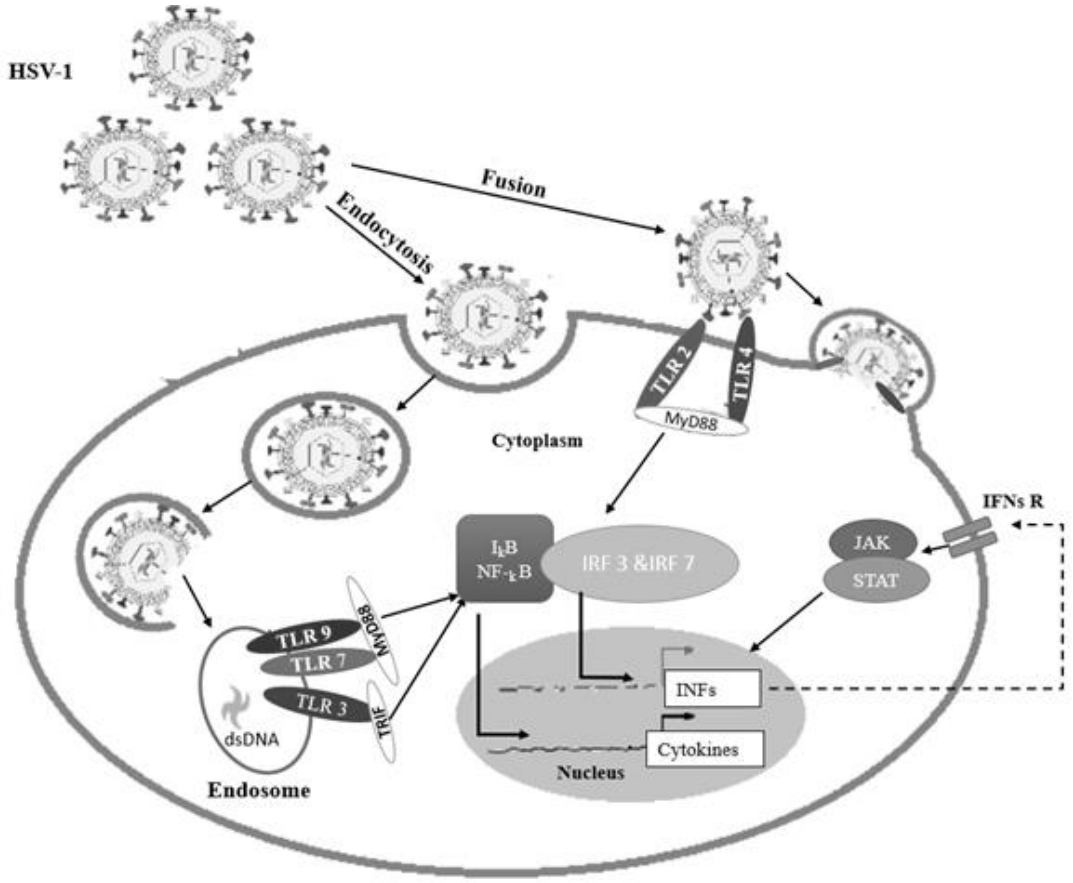


Figure 3: The Two Different Pathways for Herpes Simplex Virus Type 1 Entry into the Host Cell: by Direct Fusion within the Plasma Membrane or by the Endocytosis pathway.

LITERATURE REVIEW

Herpes Simplex Virus Type 1

The word herpes was taken from Greek ancient language meaning crawl or creep; after that herpes viruses family was named including Herpes Simplex Virus Type 1 (HSV-1) and Herpes Simplex Virus Type 2 (HSV-2) which are the most contagious pathogens in humans [16]. Herpes Simplex Virus Type 1 (HSV-1) belongs to the alphaherpesvirus subfamily, and the virion composed of four main parts including dsDNA genome (157 kb), teguments, nucleocapsid, and glycoproteins on the surface of the envelope [17]. However, it is known to infect humans and cause infectious diseases including orofacial and genital herpes [18].

HSV-1 infection is often present in tissues that are enriched in keratinocytes and nerve endings as a main target to initiate the infection. Therefore, skin, the mucosa, and the cornea are the primary entry site for the HSV-1 infection in humans [19]. In general, the virus develops two types of infection post the inoculation: primary and latent infection. Following the primary infection that occurred in the epithelial cells, the virus undergoes a latency state in ganglion cells and persists for lifelong in the victim [20]. Unfortunately, there is no treatment or vaccine that could prevent infection or eliminate latency [21].

Herpes Simplex Virus Type 1 Entry and Infection in Keratinocytes:

HSV-1 utilizes different pathways to target the host cell either by fusion with the plasma membrane directly or by endocytosis, either pH-dependent or pH-independent [22]. The infection starts when the virus encounters the skin or the mucosa and establishes binding between viral glycoproteins on the envelope and a specific type of cellular receptors to invade targeted cells on the epithelium [23]. The common receptors on the surface of the host cell that trigger HSV-1 entry are nectin-1, herpes virus entry mediator HVEM, heparan sulfate (HS), and 3-O-HS. By contrast the nectin-1 and HVEM have been reported as most involved in HSV infections and pathogenesis among humans [24].

The event of entry is initiated by binding the viral glycoproteins gB and gC to heparan sulfates (HS) and paired immunoglobulin-like type 2 receptor alpha (PILRα) on the surface of the host cell which leads gD binding to nectin-1 or HVEM and induces conformational changes on gH/gL followed by nucleocapsid fusion within the host cell plasma membrane to the cytoplasm [25]. Thus, the tegument proteins that protect the nucleocapsid, dissociate and translocate the viral genome into the nucleus of the host cell, and establish viral immediate-early transcription [26]. First, the viral immediate-early genes transcription and translation includes IE, α, E, and β followed by viral DNA replication. Then, it binds to infected cell protein 8 (ICP8) which activates the transcription and the translation of the late genes (L&γ) that were encoded for the nucleocapsid and the teguments. Eventually, the viral genome will be packaged in the nucleocapsid and released from the host cell [27-28]. Hence, the new virion is ready to invade other host cells.

In a study, it has been confirmed that the pro-inflammatory cytokine INF-β was produced in the infected epidermis keratinocytes with HSV-1 in both cell culture and specimens were taken from human patients [29]. In contrast, other study suggested that cell immune response is inhibited when viral genes are expressed that indicates the role of viral genes to inhibit antiviral response [30]. As an example, during the early phase of the infection, ICP0 is expressed and that may activate viral gene expression and inhibit type 1 IFN-gene induction resulting in augmentation of infection [31-32]. In addition, ICP0 expression may degrade interferon inducible protein-16 (IFI-16) and inhibits interferon regulatory factor-3 (IRF-3) signaling and prevents IFN type 1 induction [32].

The Role of Innate Immune System During HSV-1 Infection:

The immune system is the first line of defense in the body against pathogens and foreign substances. There are many types of immune cells involved in this duty including natural killer (NK), dendritic cells (DC), neutrophils, and macrophages; however, all these cell types participate in restricting HSV-1 invasion. Moreover, T lymphocytes including both CD4+ and CD8+ are important to produce type 1 IFN for inhibiting the HSV-1 infection and replication [33]. In contrast, HSV-1 has multiple pathways to impair anti-viral productions such as interfering with IFN expressions and functions [34].

However, the macrophages are immune cells derived from monocytes that originated from the bone marrow and migrate through the blood stream [35]. Eventually, they reside in different tissues and have different names. Hence, they play pivotal roles to protect the body from pathogens, to maintain homeostasis, to involve in damage repair, and to engulf foreign substances. Furthermore, these immune cells induce immune responses by secreting pro-inflammatory cytokines, chemokines, and tumor necrosis factors [36]. Thus, macrophages sense the danger signals via Toll-like receptors (TLRs), pattern recognition receptor (PRRs), and interleukin-1 receptor (IL-1R) and establish the MyD88 signaling pathway [13]. This binding results in pro-inflammatory cytokines as anti-viral production including IFN α/β, TNF, and IL-12.

Herpes Simplex Virus Type 1 (HSV-1) immune evasion strategies

The HSV-1 has multiple mechanisms to escape immune recognition and maintain virus replication by inhibiting innate immune cells and epithelial cells of immediate immune response soon after invasion as well as inhibiting cellular translation and programed cell death [37]. Moreover, it can interfere with the major histocompatibility complex class 1 (MHC I), complement activation, down-regulate Fc receptor, and prevent apoptosis in host cell; in addition, the HSV-1 has the ability to inhibit MHC II expression on macrophages which leads to a reduction of cytokines production by natural killer cells (NK) and T lymphocytes [38].

Likewise, the HSV-1 infected keratinocytes inhibit the pro-inflammatory cytokines such as interleukin-1β (IL-1β), and block interferon IFNs' functions [34]. By contrast HSV-1 infected keratinocytes continue to produce the interleukin-1α (IL-1α) which is important for recruiting white blood cells to the site of infection and preventing viral replication. The IL-1β shows high expression in macrophages; however, both of IL-1α and IL-1β have same cellular receptor which is IL-1 receptor type 1(IL-1R) [39]. The role of IL-1α has been demonstrated for immune cells recruitment by blocking IL-1R which lead to a significant decrease in neutrophil influx in mice [40].

MATERIALS AND METHODS

Three different cell lines and (HSV-1) virion were utilized on two models of cell culture (monolayer and co-culture system) in this study.

PAM212 is a keratinocyte cell line derived from newborn BALB/c mice and was previously obtained from Joanna Anders at NIH/NCI. The RAW 264.7 murine macrophage cell line is derived from a tumor induced by Abelson murine leukemia virus obtained from an adult male BALB/c mouse. Both are adherent cell lines by nature. They were initially thawed and seeded individually in T-25 (25 cm^2) culture flasks, and then transferred to T-75 (75 cm2) culture flasks for maintenance and cell splitting.

Each monolayer was grown in the presence of Dulbecco's Modified Eagle Medium (DMEM) (Fisher Science, Pittsburgh, PA) supplemented with 10% heat inactivated fetal bovine serum (FBS).

HSV-1 (syn17+) (initially provided by Dr. Nancy Sawtell, Children's Hospital Medical Center, Cincinnati, OH), was utilized for infection. Prior to infection, a Plaque Assay was performed to determine the virus titers in the original stock by utilizing Vero cells line (CCL-81, American Type Culture Collection) developed from African green monkey kidney epithelial cells. The PFU/ml for the virus in original stock was 6.8 X 10^7 viral particles per ml. Therefore, 0.44 µl or 0.22 µl of virus stock was added to 1000 µl media supplemented with 1% FBS per well for infection. In this study, 6-well plates were used for the cell infection and the Plaque Assay throughout the experiments. Cell cultures were incubated in a humidified incubator at 37° C, 5% CO2.

Infection Protocol for Keratinocytes (PAM-212):

The keratinocytes (PAM-212) were seeded in 6-well plates at density of 3 X 10^5 cells per well. Each well was supplied with 3 ml medium containing 10 % FBS and incubated overnight for cell adhesion. At 70% of cell confluence, old medium was discarded and washed with 2 ml of PBS prior infection. In each well, 1 ml of a new medium supplemented with 1% FBS was added. Hence, a volume of 0.44 µl was taken from the (HSV-1) original stock to infect three wells at multiplicity of infection (MOI) 0.1 ratio 1:10 while the remaining three uninfected wells were used as a negative control. Then cell cultures were incubated for 2 hours to establish the infection. In the next step, the virus was removed, cell cultures were washed with 2 ml of PBS per well, and 3 ml of fresh medium supplemented with 10% FBS was added. Again, cell cultures were incubated at 37 C^o and 5% Co_2 for 24, 48, and 72 hours after the infection. After the incubation period, the suspension was collected from the 6-well plates individually in 15 ml centrifuge tubes and centrifuged at 1600 (rpm); 4 C^o for 5 minutes. 1000 µl of the supernatant from each tube was transferred to the 1.5 ml centrifuge tubes and stored at – 80 C^o for plaque assay. The 15 ml centrifuge tubes that were used previously were reused to collect cells after trypsinization and centrifuged at 1600 (rpm); 4 C^o for 5 minutes to form a pallet in the bottom of the tubes. The supernatant obtained after trypsinization was aspirated, and the pellet was resuspended in 1 ml of 10% FBS medium for calculating the cell viability.

Infection Protocol for Unpolarized Macrophages (RAW 264.7):

Unpolarized macrophages (RAW 264.7/ M0) were seeded in 6-well plates at density of 3 X 10^5 cells per well. 3 ml of media supplemented with 10 % FBS was added to each well and incubated for cell attachment. After cell adhesion, old medium was removed and washed with 2 ml of PBS prior infection. In each well, 1 ml of a new medium supplemented with 1% FBS was added. After that, a volume of 0.44 µl was taken from the (HSV-1) original stock to infect three wells at multiplicity of infection (MOI) 0.1 ratio 1:10 while the remaining three uninfected wells used as a negative control. Then the plates were incubated for 2 hours to establish the infection. In the following step, the virus was removed, cell cultures were washed twice with 2 ml of PBS, and 3 ml of fresh medium contained 10% FBS was added. After that, cell cultures were incubated at 37 C^o and 5% Co_2 for 24, 48, and 72 hours post-infection. Following the incubation time, cells were detached form 6-well plates by utilizing cell scraper (Thermo-Fisher Scientific Brand). The suspension was collected from each well individually in 15 ml centrifuge tubes and centrifuged at 1600 (rpm); 4 C^o for 5 minutes. 1000 µl of each supernatant was transferred to the 1.5 ml Eppendorf tubes and stored at – 80 C^o for plaque assay. The pellet in the bottom of the tubes remained intact when the supernatant was taken. The remaining of supernatant was aspirated, and the pellet was resuspended in 1 ml of 10% FBS media for calculating the cell viability.

Infection Protocol for Co-culture (PAM-212& RAW 264.7):

First, keratinocytes (PAM-212) were seeded in 6-well plates at density of 1.5 X 10^5 cells per well. Each well was supplied with 3 ml medium containing 10 % FBS and incubated overnight for cell adhesion. After cell adhesion, old medium was removed and washed with 2 ml of PBS prior infection. In each well, 1 ml of a new medium supplemented with 1% FBS was added. After that, a volume of 0.22 µl was taken from the (HSV-1) original stock to infect three wells at multiplicity of infection (MOI) 0.1 ratio 1:10 while the remaining three uninfected wells were used as a negative control. Then the plates were incubated for 2 hours to initiate cell infection. In the following step, the virus was removed, cell cultures were washed with 2 ml of PBS, and 3 ml of fresh media containing 10% FBS was added. Second, unpolarized macrophages were added at ratio 1:5 of keratinocytes [1]. Thus 3 X 10^4 cells of M0 macrophages (RAW264.7) were added to the 1.5 X 10^5 cells of keratinocytes (PAM-212) previously seeded and infected with HSV-1 in 6-well plates. After that, cell cultures were incubated at 37 C^o and 5% CO_2 for 24, 48, and 72 hours post-infection. Following the incubation period, the suspension was collected from each well individually in 15 ml centrifuge tubes and centrifuged at 1600 (rpm); 4 C^o for 5 minutes. 1000 µl of the supernatant of each was transferred to the 1.5 ml Eppendorf tubes and stored at – 80 ^{o} C for plaque assay. The 15 ml centrifuge tubes that were used previously, were reused again to collect cells after trypsinization and centrifuged at 1600 (rpm); 4 C^o for 5 minutes to form a pallet in the bottom of the tubes. The supernatant obtained after trypsinization was aspirated, and the pellet was resuspended in 1 ml of 10% FBS media for calculating the cell viability.

Cell Viability:

Trypan Blue Exclusion Test was used to determine the cell viability which indicates the percentages of viable and non-viable cells in the suspension obtained from cell culture. It requires a sample of suspension, hemocytometer, trypan blue stain (Fisher Scientific, Pittsburgh, PA brand), coverslips, micropipettes (P1000, P200, and P20), blue and yellow tips, and light microscope. First, the sample of suspension was obtained by scraping and/or trypsinization as described previously. The suspension was collected in 15 ml centrifuge tubes and centrifuged at 1600 (rpm); 4 C^o for 5 minutes to form a pellet in the bottom of the tubes. The supernatant was aspirated, and the pellet was resuspended in 1 ml of 10% FBS media. Second, cells were mixed well utilizing a micropipette (P1000). Then, 100 µl of trypan blue stain and 50 µl of the suspension were transferred via micropipette (P200) to 1.5 ml Eppendorf tube and mixed genially. Third, 10 µl was taken by micropipette (P20) form the blend and placed on the hemocytometer initially covered with coverslip. Fourth, the hemocytometer was examined under the 10x magnification power in the light microscope. Finally, cells were counted, viable cells were remained unstained while non-viable cells were stained blue.

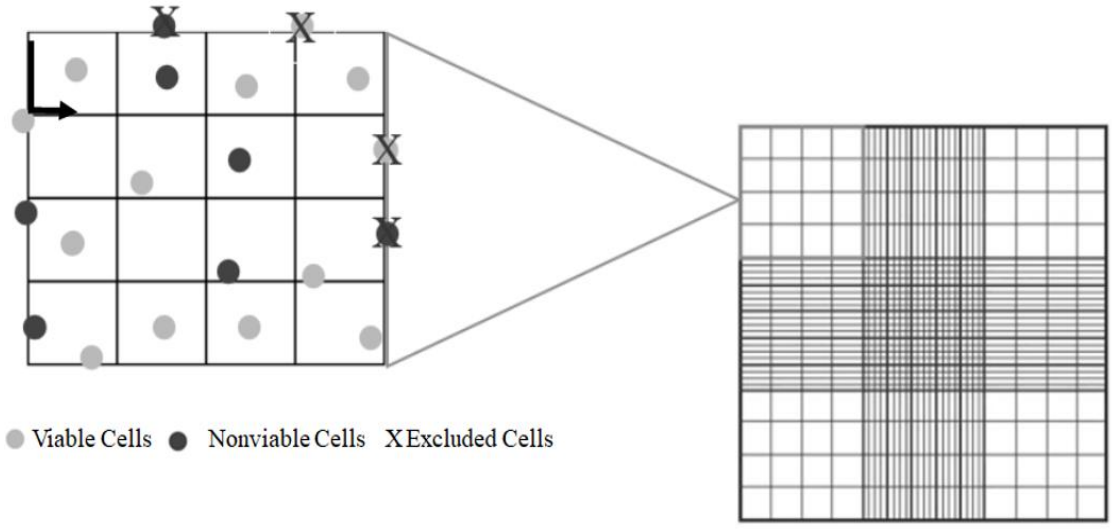


Figure 4: Trypan Blue Exclusion Test utilizing hemocytometer.

Under the light microscope lens at 10x magnification power, cells were counted. Each circular dot represents cell. The bright dots represent viable cells while the dark once represent non-viable cells. L shaped arrow shows pathway how cells were counted.

Plaque Assay:

The plaque assay measures infectious virus particles contained in a stock. Thus, plaque formation means the virus was able to initiate infection, propagate and display cytopathic effect (CPF) on cell culture. The plaques can be counted and visualized by the naked eye after staining the plate with 1% of crystal violet as colorless spots. Therefore, virus titers were calculated based on the plaque forming units per ml (PFU/ml).

Herpes Simplex Virus-1 strain Syn 17+ was previously obtained from (Dr. Nancy Sawtell, Children's Hospital Medical Center, Cincinnati, OH). Prior to utilizing HSV in this study for infection, virus titers were determined. Plaque assay was performed on Vero cells at 100% confluency. Initially, Vero cells were seeded in 6-well plates at density of 3 X 10^5 cells per well. 3 ml of media supplemented with 10 % FBS was added to each well. Vero cells usually reach 100% confluency within 2-3 days. Before infection, a serial dilution for original stock is required (Figure 4). First, 10 - 1.5 ml centrifuge tubes were set on a rack and labeled from -1 to -10 as a dilution factor. Then 900 µl of DMEM without FBS was added to each tube. A 100 µl of virus stock was transferred to the first tube. Next, 100 µl was taken from tube -1 to tube -2, this process continued to the last tube -10. After that, old medium was removed from Vero cells culture and washed with 2 ml of PBS prior infection. To infect cells, 500 µl of diluted virus was added to each well. The first well of 6-well plates was used as a negative control. Then, the plates were incubated for 1 hour to establish the infection. In the following step, the virus was removed, cell cultures were washed once with 2 ml of PBS and covered with 3 ml of 4% methyl-cellulose. Next, plates were incubated at 37 C^o and 5% Co_2. The plaques were usually seen after 3-5 days. After the plaques were formed, 3

ml of 4% paraformaldehyde was added for fixation. Plates were incubated at room temperature for 1 hour. Then agarose and paraformaldehyde were removed and washed gently with tap water. About 2 ml of 1% crystal violet solution (Sigma) was added and incubated at room temperature for 5-10 minutes. Stain was removed with tap water, and plaques were counted as followed.

$$\text{PFU/ml (of original stock)} = \frac{\text{number of plaques}}{\text{dilution factor x (ml on inoculum /plate)}}$$

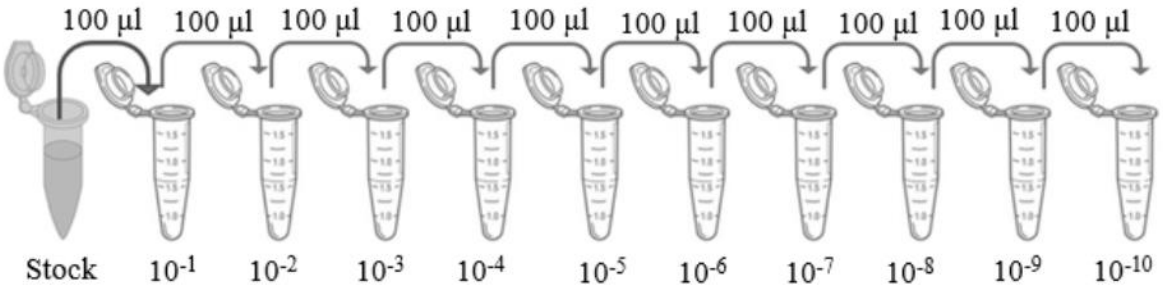


Figure 5: Serial Dilution Method for Plaque Assay.

Statistical Analysis

Data were obtained from conducting triplicates within all experiments in this study. Sigma-Plot 12.0 software; One-Way ANOVA, was utilized for analyzing data and calculating statistical significance. Outcomes were compared for the P-values, any data showing ≤ 0.05 were considered as statistically significant.

Symbol	Meaning
ns	$P > 0.05$
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$

Table 1: Symbol Representation and Meaning for Indicating the Statistical Difference.

RESULTS

Cell Viability:

The Cell Viability of Uninfected and Infected Keratinocytes (PAM-212) with HSV-1 after 24, 48, and 72 Hours.

Keratinocytes were either uninfected (control) or infected with herpes simplex virus type 1 at multiplicity of infection (MOI) 0.1. Cell viability was determined after 24, 48, 72 hours by performing Trypan Blue Exclusion Test. In all experiments exhibited significant decrease in cell viability at 24, 48, and 72 hours post infection. Keratinocytes exhibited slightly significant decrease (~6.5%, P-value = 0.001) in cell viability following infection with HSV-1 compared to control at 24 hours. Also, a significant decrease in viability was observed in keratinocytes (~16.6%, P-value = < 0.001) following infection at 48 hours. However, after 72 hours of infection there was a strongly significant decrease in cell viability about (~46.7%, P-value = <0.001). In contrast, there was no significant difference between in infected keratinocytes at 24 and 48 hours (P-value = 0.055). In contrast, keratinocytes infected with HSV-1 at 72 hours showed significant decrease in cell viability compared to 24 and 48 hours (P-value = <0.001, P-value = <0.001) respectively (Figure 6).

The Cell Viability of Uninfected and Infected Macrophages (RAW 264.7) with HSV-1 after 24, 48, and 72 Hours.

Macrophages were either uninfected (control) or infected with herpes simplex virus type 1 at multiplicity of infection (MOI) 0.1. Cell viability was counted after 24, 48, and 72 hours by utilizing Trypan Blue Exclusion Test. There was no statistically significant difference in cell viability at 24 post infection (~1%, P-value = 0.439). At 48 and 72 hours following infection, macrophages exhibited a large significant decrease in cell viability (~8.1%, 46.3%, P-value = <0.001, P-value = <0.001), respectively, compared to control. There was significant difference between infected macrophages at 24 and 48 hours (~5.3 %, P-value = 0.033). In contrast, at 72 hours macrophages infected with HSV-1 showed significant decrease in cell viability compared to 24 and 48 hours (~44.8%, ~39.5%, P-value = <0.001, P-value = <0.001) respectively (Figure 7).

The Cell Viability of Uninfected and Infected Co-culture (RAW 264.7 & PAM-212) with HSV-1 after 24, 48, and 72 Hours.

In the co-culture model, keratinocytes were first either infected with HSV-1 or uninfected as a negative control at multiplicity of infection (MOI) 0.1 and incubated for 2 hours, then the macrophages were added at ratio of 1:5 and incubated for time-points. Cell viability was examined after 24, 48, and 72 hours by utilizing Trypan Blue Exclusion Test. They exhibited a slightly significant difference in cell viability at 24 post infection (~1% (*) P-value = 0.026). While at 48 and 72 hours following infection, co-cultured cell lines exhibited a large significant decrease in cell viability (~36.3%, 64.6%, P-value = <0.001, P-value = <0.001), respectively compared to the control. However; in comparison to the different of time-points, there was a significant difference between infected co-cultured cell lines at 24, 48 and 72 hours (P-value = <0.001, P-value = <0.001, and P-value = <0.001) respectively (Figure 8).

The Cell Viability compared between all Cell Lines (RAW 264.7, PAM-212, and Co-culture) Uninfected and Infected with HSV-1 after 24 Hours.

The cell viability was tested for all cell lines (RAW 264.7/M0 & PAM-212) in monolayers and co-culture model, after they were infected with herpes simplex virus type 1 at multiplicity of infection (MOI) 0.1 and incubation time for 24 hours. The cell lines were either infected with HSV-1 or uninfected as a negative control. Cell viability was examined after 24 hours by utilizing Trypan Blue Exclusion Test. The keratinocytes (PAM-212) monolayer exhibited a significant reduction in cell viability at 24 post infection (~6.5%, P-value = 0.001). The macrophage (RAW 264.7) monolayer showed no significant difference at 24 hours following infection. By contrast the co-cultured cell lines exhibited a little significant decrease in cell viability (~1%, P-value = 0.026) compared to the control. However, in comparison between the co-cultured cell lines versus the different monolayers cell line, it showed a significant increase in cell viability at 24 hours (~7.7%, 7.5%; P-value = <0.001, P-value = <0.001) respectively. There was no significant difference between the keratinocytes and macrophages M0 monolayers (~ 0.002%, P-value = 0.920) (Figure 9).

The Cell Viability compared between all Cell Lines (RAW 264.7/M0, PAM-212, and Co-culture) Uninfected and Infected with HSV-1 after 48 Hours.

The cell viability was tested for all cell lines (RAW 264.7/M0 & PAM-212) in monolayers and co-culture model, after they were infected with herpes simplex virus type 1 at multiplicity of infection (MOI) 0.1 and incubated for 48 hours. The cell lines were either infected with HSV-1 or uninfected as a negative control. Cell viability was examined after 48 hours by utilizing Trypan Blue Exclusion Test. All monolayers and co-cultured cell lines (PAM-212, RAW 264.7/M0, and Co-culture) exhibited a significant decrease in cell viability at 48 post infection (~16.6%, 8.1%, & 36.3%; P-value = <0.001 P-value = <0.001, P-value = <0.001) respectively were compared to control. However; in comparison between the different of cell lines, there was no significant difference between the keratinocyte and macrophage monolayers infected groups (~5.1 %, P-value = 0.282) while the co-cultured cell lines infected group showed a great significant decrease in cell viability at 48 hours (~19 %, 24.1%; P-value = <0.001, P-value = <0.001) compared to (PAM-212 & RAW 264.7/M0) respectively (Figure 10).

The Cell Viability compared between all Cell Lines (RAW 264.7, PAM-212, and Co-culture) Uninfected and Infected with HSV-1 after 72 Hours.

The cell viability was tested for all cell lines (RAW 264.7/M0 & PAM-212) in monolayers and co-culture model, after they were infected with herpes simplex virus type 1 at multiplicity of infection (MOI) 0.1 and incubated for 72 hours. The cell lines were either infected with HSV-1 or uninfected as a negative control. Cell viability was examined after 72 hours by utilizing Trypan Blue Exclusion Test. All monolayers and co-cultured cell lines (PAM-212, RAW 264.7/M0, and Co-culture) exhibited a great significant decrease in cell viability at 72 post infection (~46.7%, 46.3%, & 64.6%; P-value = <0.001 P-value = <0.001, P-value = <0.001) respectively. However, there was no significant difference between the keratinocytes and macrophages M0 monolayers infected groups (~6.1%, P-value = 0.323) while the co-cultured cell lines infected group showed a great significant decrease in cell viability at 72 hours (~26.7 %, 20.6%; P-value = <0.001, P-value = 0.003) compared to (PAM-212 & RAW 264.7) respectively (Figure 11).

Herpes Simplex Virus Type1 Cytopathic Effect (CPE):

All cell lines (PAM-212, RAW 264.7, and co-culture) were seeded, maintained, and infected on 6-well plates. In each plate, three wells were infected with HSV-1 and the three remaining were used as a negative control. Post-infection, plates were incubated at 37C^{o}, 5% $Co_{2,}$ for 24, 48, and 72 hours. The HSV-1 cytopathic effect (CPE) was evident in all infected groups during incubation time-points. After 24 hours of infection, some of cells appeared enlarged in size, irregularly shaped, and some were floating in the medium, that indicates cell lysis and HSV-1 cytopathic effect. At 48 post-infection, same signs were observed, but in higher percentages. Furthermore, at 72 hours post-infection cell were mostly detached and more debris were visible. The HSV-1 cytopathic effect (CPE) on Vero cell was clear prior to plaque formation (Figure 12).

Plaque Assay:

Plaque assay was utilized to calculate HSV-1 concentrations in different samples were collected from different experiments and stored at – 80 C^{o}. Therefore, Plaque assay was performed in triplicate on 6-well plates, and each single assay represents different samples within experiment. The supernatant that used for plaque assay was obtained from keratinocytes (PAM-212), macrophages (M0), and (PAM-212 and M0) co-cultured cell lines post-infected them with HSV-1 at 24, 48, and 72 hours. Plaques formation were evident at different serial dilution factors (SDFs) from 10^{-1} to 10^{-10}.

The Plaque Assay for Keratinocytes (PAM-212) Infected with HSV-1 at 24, 48, and 72 Hours.

In keratinocytes (PAM-212) at 24 hours, plaques formation was observed from 10^{-1} to 10^{-5}. However, the virus titers were counted at $x10^{-3}$, and the plaque formed per ml (PFU/ml) was 12.6 X 10^{-3} in average. At 48 hours, plaques formation was observed from 10^{-1} to 10^{-10}. The virus titers were counted at $x10^{-8}$, and the plaque formed per ml (PFU/ml) was 214 X 10^{-8} in average. While at 72 hours, plaques formation was observed from 10^{-1} to 10^{-10}. The virus titers were counted at $x10^{-8}$, and the plaque formed per ml (PFU/ml) was 224 X 10^{-8} in average. On the other hand, HSV-1 titers were significantly increased in the keratinocytes from 24 to 48 and 72 hours respectively. In contrast, there was no significant difference between virion replication at 48 and 72 hours (Figure 13).

The Plaque Assay for Macrophages (RAW 264.7 / M0) Infected with HSV-1 at 24, 48, and 72 Hours.

In macrophages (RAW 264.7/ M0) at 24 hours, plaque formation was observed from 10^{-1} to 10^{-5}. However, the virus titers were counted at $x10^{-3}$, and the plaque formed per ml (PFU/ml) was 65.6 X 10^{-3} in average. At 48 hours, plaques formation was only observed from 10^{-1} to 10^{-5} and there were no plaques formation beyond the dilution factor $x10^{-5}$ as was demonstrated by six trials. The virus titers were counted at $x10^{-2}$, and the plaque formed per ml (PFU/ml) was 132 X 10^{-2} in average. While at 72 hours, plaques formation was observed from 10^{-1} to 10^{-10}. The virus titers were counted at $x10^{-8}$, and the plaque formed per ml (PFU/ml) was 91 X 10^{-8} in average. Moreover, HSV-1 titers were remarkably decreased in the macrophages from 24 to 48 hours. However, there was a large significant increase in virion titers at 72 hours compared to 24 and 48 hours (Figure 14).

The Plaque Assay for Co-culture (PAM-212 + M0) Infected with HSV-1 at 24, 48, and 72 Hours.

In co-culture model (PAM-212 + RAW 264.7/M0) at 24 hours, plaque formation was observed only at 10^{-1} in one plate while other plate did not show any plaque formation beyond the dilution factor $x10^{-1}$ as was demonstrated by four trials. However, the virus titers were counted at $x10^{-1}$, and the plaque formed per ml (PFU/ml) was 55.3 X 10^{-1} in average. At 48 hours, plaque formation was observed from 10^{-1} to 10^{-10}. The virus titers were counted at $x10^{-8}$, and the plaque formed per ml (PFU/ml) was 34.6 X 10^{-8} in average. While at 72 hours, plaques formation was observed from 10^{-1} to 10^{-10}. The virus titers were counted at $x10^{-8}$, and the plaque formed per ml (PFU/ml) was 91.3 X 10^{-8} in average. However, HSV-1 titers were noticeably decreased in the co-culture model at 24 compared to virus titers at 48 and 72 hours respectively. Nevertheless, there was a slight difference between virion concentrations at 48 and 72 hours (Figure 15).

Time / Cell Lines	Keratinocytes (PAM-212) PFU/ml	Macrophages (RAW 264.7) PFU/ml	Co-culture Model (PAM-212 + RAW 264.7) PFU/ml
24 Hours	12.6×10^{-3}	65.6×10^{-3}	55.3×10^{-1}
48 Hours	214×10^{-8}	132×10^{-2}	34.6×10^{-8}
72 Hours	224×10^{-8}	91×10^{-8}	91.3×10^{-8}

Table 2: This table illustrates Plaque Forming Units Per ml (PFU/ml) for Herpes Simplex Virus Type 1 Obtained by Plaque Assay.

The Plaque Assay compared between all Cell Lines (RAW 264.7, PAM-212, and Co-culture) Infected with HSV-1 after 24 Hours.

The plaque assay was performed for all the infected cell lines (RAW 264.7/M0 & PAM-212) in monolayers and co-culture model. Then, the HSV-1 titers were compared for 24 hours. The keratinocytes (PAM-212) monolayer exhibited less significant virus concentration compared with macrophages at 24 hours (~5.1fold, P-value = <0.001). The macrophage (RAW 264.7 /M0) monolayer showed the most significant increase in HSV-1 replication at 24 hours compared to keratinocytes and co-culture (~5.1 fold, 118 fold, P-value = <0.001, P-value = <0.001) respectively. On the other hand, the co-cultured cell lines (PAM-212 + RAW 264.7/M0) exhibited the lowest significant in virion titers compared to the monolayers infected groups at 24 hours (~22.7 fold, 118 fold, P-value = <0.001, P-value = <0.001) respectively (Figure 16).

The Plaque Assay compared between all Cell Lines (RAW 264.7, PAM-212, and Co-culture) Infected with HSV-1 after 48 Hours.

The plaque assay was performed for all the infected cell lines (RAW 264.7/M0 & PAM-212) in monolayers and co-culture model. Then, the HSV-1 titers were compared for 48 hours. The keratinocytes (PAM-212) monolayer exhibited no significant in virus concentration compared with co-culture at 48 hours (~1.5 fold, P-value = 0.078). While the macrophages (RAW 264.7 /M0) monolayer showed the lowest significant in HSV-1 replication at 48 hours compared to keratinocytes and co-culture (~$1.65X10^6$ fold, $1.11X10^6$ fold, P-value = 0.008, P-value = 0.012) respectively (Figure 17).

The Plaque Assay compared between all Cell Lines (RAW 264.7, PAM-212, and Co-culture) Infected with HSV-1 after 72 Hours.

The plaque assay was performed for all the infected cell lines (RAW 264.7/M0 & PAM-212) in monolayers and co-culture model. Then, the HSV-1 titers were compared for 72 hours. The keratinocytes (PAM-212) monolayer exhibited a significant increase in virus concentration compared with macrophages (M0) and co-culture at 72 hours (~5.6 fold, 2.44 fold, P-value = <0.001, P-value = <0.001). While the macrophages (RAW 264.7 /M0) monolayer showed the less significant in HSV-1 replication at 72 hours compared to keratinocytes and co-culture (~5.6 fold, 2.3 fold, P-value = <0.001, P-value 0.052) respectively (Figure 18).

DISCUSSION

The goal of this study was to develop a new model of cell co-culture that could illustrate the role of the macrophages (RAW 264.7) in responding to Herpes Simplex Virus Type 1 (HSV-1) within infected keratinocytes. For this purpose, we used macrophages (RAW 264.7) and murine keratinocytes (PAM-212) to investigate the susceptibility of each cell line in monolayer to the HSV-1 infection. In the next step, we started to infect the keratinocytes with HSV-1 for two hours at the multiplicity of infection (MOI) 0.1. Then the virus was removed, and the cell culture was washed with PBS prior to co-culturing them with the macrophages at ratio "1:5" [1] for time-points of 24, 48, and 72 hours. In this study, we just focused on the cytopathic effect (CPE), cell viability, and plaque assay.

Interestingly, we found that there were some differences and similarities between keratinocytes (PAM-212) and macrophages (RAW 264.7) in all the models that we used in this study. We observed some morphology changes that were a result of the HSV-1 invasion to the host cells after 24, 48, and 72 hours. These morphology changes illustrate the cytopathic effects (CPE) of the HSV-1 infection. However, the different cell lines that we utilized in this project exhibited almost the same morphology changes in the earlier phase of the infection at 24 and 48 hours. The infected cells appeared either irregularly shaped or rounded cells, enlarged in size, and seemed in different density compared to the surrounding area. In contrast, at 72 hours cells were degraded and detached, and there was more debris in the medium in the keratinocytes monolayer. The macrophage monolayers and the co-culture were similar to keratinocytes except the medium was clearer with less debris (Figure 12).

We attributed the clearance of debris in the medium to the macrophages' ability to engulf and digest the foreign substance. Same signs of cytopathic effects have been observed in vitro on the infected epithelium and described as "a reticular degeneration and ballooning of the cells as well as the appearance of large irregular or multinucleated giant cells" [41-42].

Our results suggest that the PAM-212 and RAW 264.7 cell lines were susceptible to the HSV-1 infection and replication within the observation time-points of 24, 48, and 72 hours. In spite of the fact that macrophages are more likely to play pivotal role in preventing the HSV-1 infection, they were found also to be susceptible to the virus replication [35].

The results of the cell viabilities were significantly decreased in the keratinocytes PAM-212 monolayer infected with HSV-1 at 24 hours, while the infected macrophages showed no significant difference in cell viability at 24 hours post-infection. That shows both PAM-212 and RAW 264.7 susceptible to the virion. On the other hand, in the co-culture model there was a noticeable increase in cell viability of the PAM-212 keratinocytes at 24 hours. Since the macrophages were added two hours later to the infected PAM-212, they response to the stimuli produced from the infected keratinocytes. That suggests the role of macrophages in preventing pathogens invasion in the body and in the co-culture model.

Surprisingly, at 24 hours post-infection our results of the plaque assay show a significant increase in the virus concentration (~5.1 fold, P-value = <0.001) in the macrophages supernatant compared to the PAM-212 which exhibited less virus titers

that suggests the ability of HSV-1 to suppress the immune response at an early phase of the infection (Figure 16). These results are in agreement to what has been reported in the ability of the HSV-1 to manipulate the immune invasion strategies to maintain virus replication in epithelial cells and innate immune cells [37].

In our co-culture model, we assumed that the role of macrophages that restrict HSV-1 dissemination. Our results demonstrate a remarkable decrease in the virus PFU/ml in this model at the first 24 hours compared to the keratinocytes and the macrophages monolayers (~22.7 fold, 118 fold, P-value = <0.001, P-value = <0.001) respectively (Figure 16). Therefore, we attribute that to the immune response of RAW 264.7 macrophages to virus released from the keratinocytes infected with the HSV-1 two hours prior to the addition of macrophages at ratio 1:5 (macrophages : keratinocytes). Thus, we think that the cytokines which were produced from infected cells such as INF-α/β, IL-1α, TNF, and reactive oxygen species (ROS) activate the macrophage and guide them toward the site of infection. Moreover, it has been reported that the interleukin-1β was inhibited by the HSV-1 infection and block IFNs' function; on the other hand, the interleukin-1α is released from the host cell, and that plays role in recruiting leucocytes to the site of infection by expressing the IL-1 receptor type 1(IL-1R1) which is the same in both the epithelial cells and macrophages [30-35]. Therefore, we attribute the increase of cell viability and the decrease of virus titers at 24 hours in co-culture to the stimulus which could be IL-1α.

However, the cell viability was significantly decreased in the PAM-212 and macrophages monolayer, and the co-culture model infected with HSV-1at 48 hours post-infection. There was no significant difference in cell viability between the

infected groups in RAW 264.7 and PAM-212 at 48 hours post-infection compared to the co-culture (Figure 11). We think that the decrease in macrophage cell viability due to the increase of pro-inflammatory cytokines production such as TNF-α which is known by its cytotoxicity to the cells, but it plays role in immune response against invaders. Also, in the co-culture model was decreased in cell viability due to the duration of exposure to the virus.

In the plaque assay result at 48 hours post the HSV-1 infection, the macrophages monolayer showed a sharp decrease in the virus titers compared to the keratinocytes which increase the virion concentration that counted on the plaque forming units per ml. Our results suggest that the macrophages monolayer was able to induce an immune response and restrict the HSV-1 replication during the incubation period of 48 hours compared to 24 hours (~ 4.9 fold, P-value = <0.001) (Figure 14). In contrast, the keratinocyte monolayer and co-culture exhibited a significant increase in the virus progeny (~$1.65X10^6$ fold, $1.11X10^6$ fold, P-value = 0.008, P-value = 0.012) respectively (Figure 17).

Our experiment outcomes show a significant decrease the cell viability in the PAM-212 and macrophages monolayer, and the co-culture model infected with HSV-1at 72 hours post-infection, but however, there was no significant difference in cell viability between the infected groups in RAW 264.7 and PAM-212 at 72 hours post-infection compared to the co-culture (Figure 11). We think that the decrease in cell viability in all cell line models due to many factors includes the increase of cytokines cytotoxicity, the increase in virus titers, the duration of exposure, and/or macrophages depletion especially in the co-culture model.

Finally, we have noticed in the plaque assay results that the macrophages were unable to withstand with the virus replication at 72 hours. That may be due to the increase of pro-inflammatory cytokines level such as TNF-α which is known by its cytotoxicity to the cells as a response to the infection. Therefore, the macrophages were depleted, and their function was impaired that permitted the virus dissemination. However, in our results of the macrophages still show a decrease in the virus titers compared to the keratinocytes monolayer and there was no significant difference between the virus concentration in the macrophages and the co-culture at 72 hours (~5.6 fold, 2.3 fold, P-value = <0.001, P-value = 0.052) respectively (Figure 18).

Future Studies:

In a real-life situation, macrophages along with other innate immune cells would should restrict HSV-1 invasion by induction of antiviral response. This in vitro co-culture study system does not permit infiltration of immune cells such as neutrophils, dendritic cells (DC), and macrophages, a limitation of the study. Further studies in understanding the immune response of macrophages (RAW 264.7) to HSV-1 infected keratinocytes (PAM-212) would include identification of the cytokines and other molecules involved, especially IFN-α/β, TNF-α, IL1-α/β, IL- 10, and ROS. In addition, this co-culture model could be altered to simulate the effect of macrophages infiltration by adding additional macrophages at ratio 1:5 or 2:5 (macrophages to keratinocytes) two hours after initiating co-culture. Another way to alter the co-culture system is to enhance virus clearance by adding M1 macrophages at 2 hours after initiation of co-culture; this should enhance the anti-viral effect of IFN-β and other ant-viral macrophage products in the system.

The results from this co-culture system showed a significant increase in cell viability and a noticeable decrease in HSV-1 titers at 24 hours suggest a role of IL-1α and early production of IFN-β. Presumably, the IL-1α produced by the keratinocytes two hours after HSV-1 infection stimulates macrophages to produce IFN-β. Because IL-1α is produced by both macrophages and keratinocytes, blocking IL-1 receptor type 1(IL-1R) with specific antibody should interfere with production of antiviral IFN-β and allow greater virus production. Based on this conjecture, a mixture of IFN-β and IL-1α may be considered as a potential therapy for herpes simplex virus Type 1 (HSV-1) infection.

Figures

The Cell Viability of Uninfected and Infected Keratinocytes (PAM-212) with HSV-1 after 24, 48, and 72 Hours

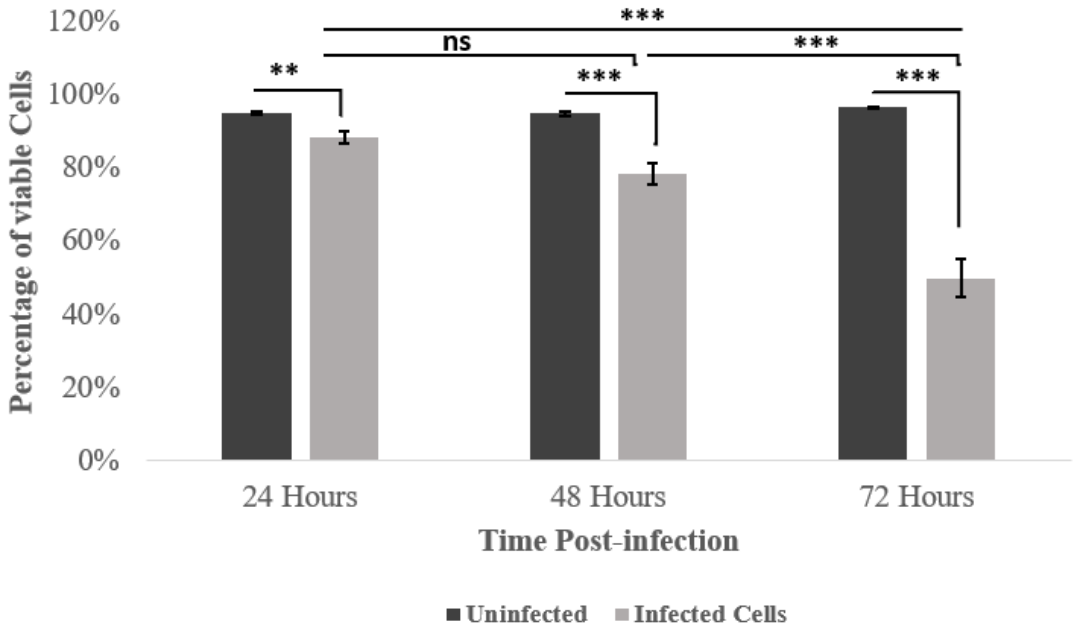


Figure 6: Cell Viability obtained by Trypan Blue Exclusion Test utilizing hemocytometer for Keratinocytes (PAM-212) after 24, 48, and 72 hours.

Keratinocytes exhibited slightly significant decrease (~6.5%, P-value = 0.001) in cell viability following infection with HSV-1 compared to control at 24 hours. Also, a significant decrease in viability was observed in keratinocytes (~16.6%, P-value = < 0.001) following infection at 48 hours. However, after 72 hours of infection there was a great significant decrease in cell viability about (~46.7%, P-value = <0.001). By comparison to the different of all time-periods, there was no significant difference between in infected keratinocytes at 24 and 48 hours (P-value = 0.055). In contrast, keratinocytes infected with HSV-1 at 72 hours showed significant decrease in cell viability compared to 24 and 48 hours (P-value = <0.001, P-value = <0.001) respectively. The mean illustrates the percentages of viable cells and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level **, p≤ 0.01; ***, p ≤ 0.001, ns = no significant (n= 3).

The Cell Viability of Uninfected and Infected Macrophages (RAW 264.7) with HSV-1 after 24, 48, and 72 Hours

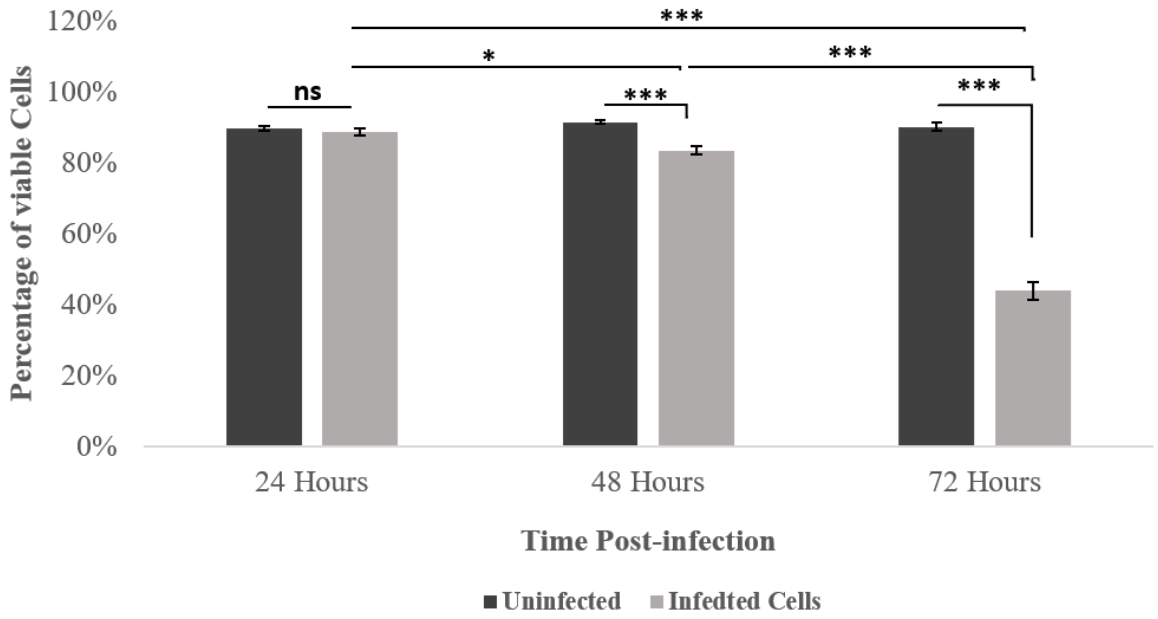


Figure 7: Cell Viability obtained by Trypan Blue Exclusion Test utilizing hemocytometer for Macrophages (RAW 264.7) at 24, 48, and 72 hours.
Macrophages showed no a statistically significant difference in cell viability at 24 post infection (~1%, P-value = 0.439). While at 48 and 72 hours following infection, macrophages exhibited a large significant decrease in cell viability (~8.1%, 46.3%, P-value = <0.001, P-value = <0.001) respectively compared to control. In comparison to the different of time-periods, there was significant difference between infected macrophages at 24 and 48 hours (~5.3 %, P-value = 0.033). In contrast, macrophages infected with HSV-1 at 72 hours showed significant decrease in cell viability compared to 24 and 48 hours (~44.8%, ~39.5%, P-value = <0.001, P-value = <0.001) respectively. The mean illustrates the percentages of viable cells and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level, *, $P \leq 0.05$, ***, $p \leq 0.001$; ns, $P > 0.05$ = no significance. (n= 3)

The Cell Viability of Uninfected and Infected Co-culture (RAW 264.7& PAM-212) with HSV-1 after 24, 48, and 72 Hours

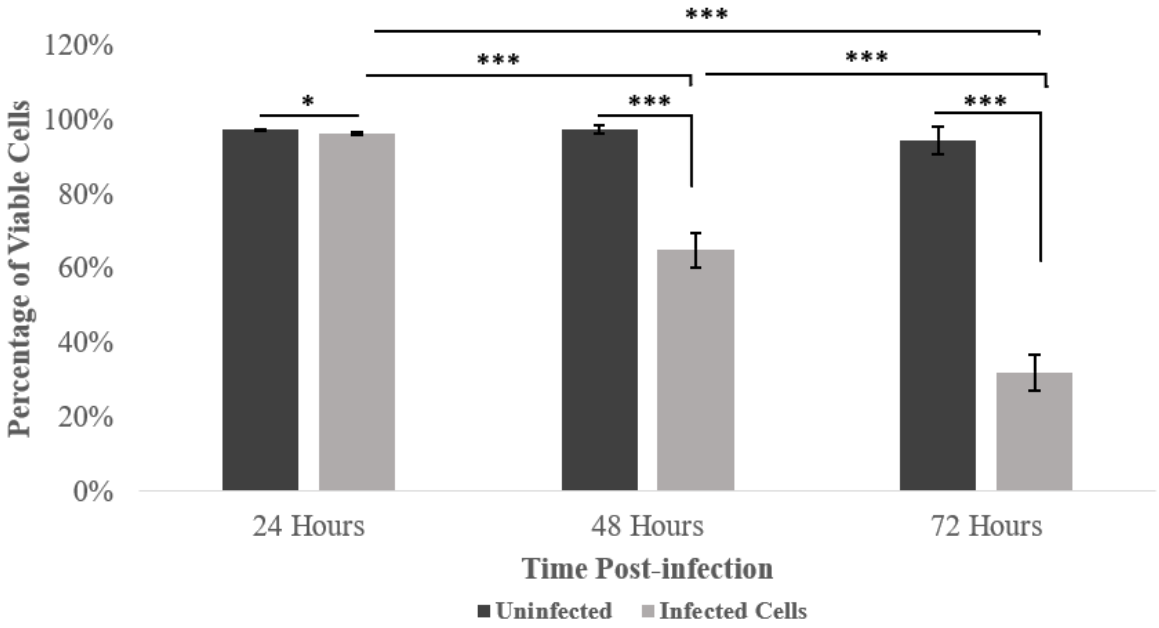


Figure 8: Cell Viability obtained by Trypan Blue Exclusion Test utilizing hemocytometer for Co-culture (RAW 264.7 PAM-212) at 24, 48, and 72 Hours post-infection with HSV-1.

In the co-culture model exhibited a slightly significant difference in cell viability at 24 post infection (~1% (*) P-value = 0.026). While at 48 and 72 hours following infection, co-cultured cell lines exhibited a large significant decrease in cell viability (~36.3%, 64.6%, P-value = <0.001, P-value = <0.001) respectively compared to the control. However; in comparison to the different of time-points, there was a grate significant difference between in infected co-cultured cell lines at 24, 48 and 72 hours (P-value = <0.001, P-value = <0.001, and P-value = <0.001) respectively. The mean illustrates the percentages of viable cells and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level,*, $p \leq 0.05$; ***, $p \leq 0.001$. (n= 3)

The Cell Viability of Uninfected and Infected (PAM-212, RAW 264.7, and Co-culture) with HSV-1 after 24 Hours

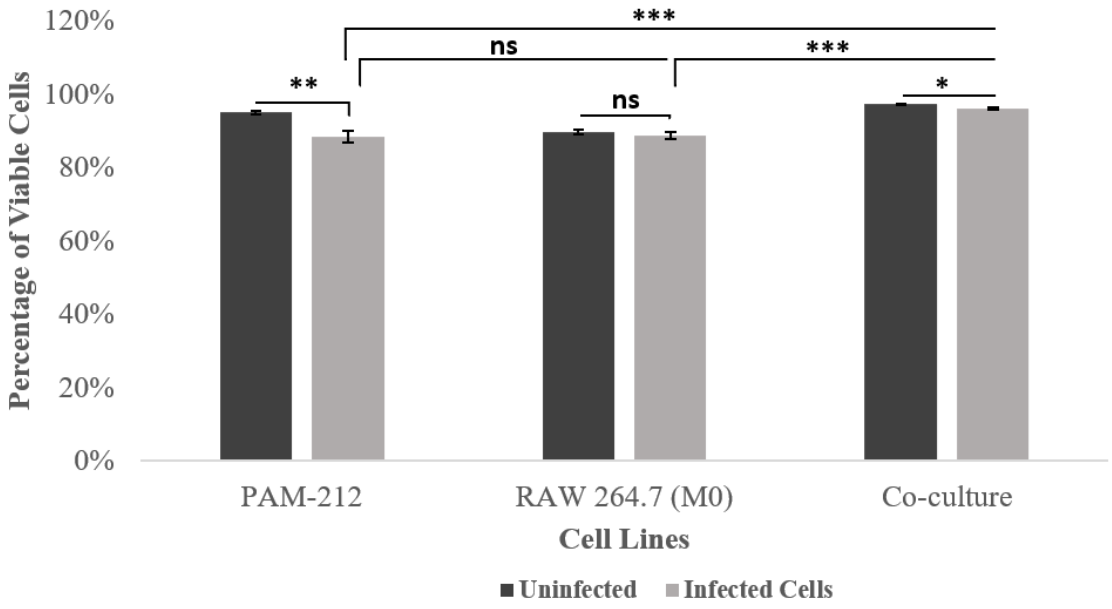


Figure 9: Cell Viability obtained by Trypan Blue Exclusion Test utilizing hemocytometer for (RAW 264.7, PAM-212, and Co-culture) at 24 Hours post-infection with HSV-1.

The keratinocytes (PAM-212) monolayer exhibited a significant reduction in cell viability at 24 post infection (~6.5%, P-value = 0.001). While the macrophages (RAW 264.7 /M0) monolayer showed no significant difference at 24 hours following infection. Whereas, the co-cultured cell lines exhibited a little significant decrease in cell viability (~1%, P-value = 0.026) compared to the control. However, in comparison between the co-cultured cell lines versus the different monolayers cell line, it showed a grate significant increase in cell viability at 24 hours (~7.7%, 7.5%; P-value = <0.001, P-value = <0.001) respectively. While there was no significant difference between the keratinocytes and macrophages M0 monolayers (~ 0.002%, P-value = 0.920). The mean illustrates the percentages of viable cells and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level, *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $p \leq 0.001$; ns, $P > 0.05$ = no significance. (n= 3)

The Cell Viability of Uninfected and Infected (PAM-212, RAW 264.7, and Co-culture) with HSV-1 after 48 Hours

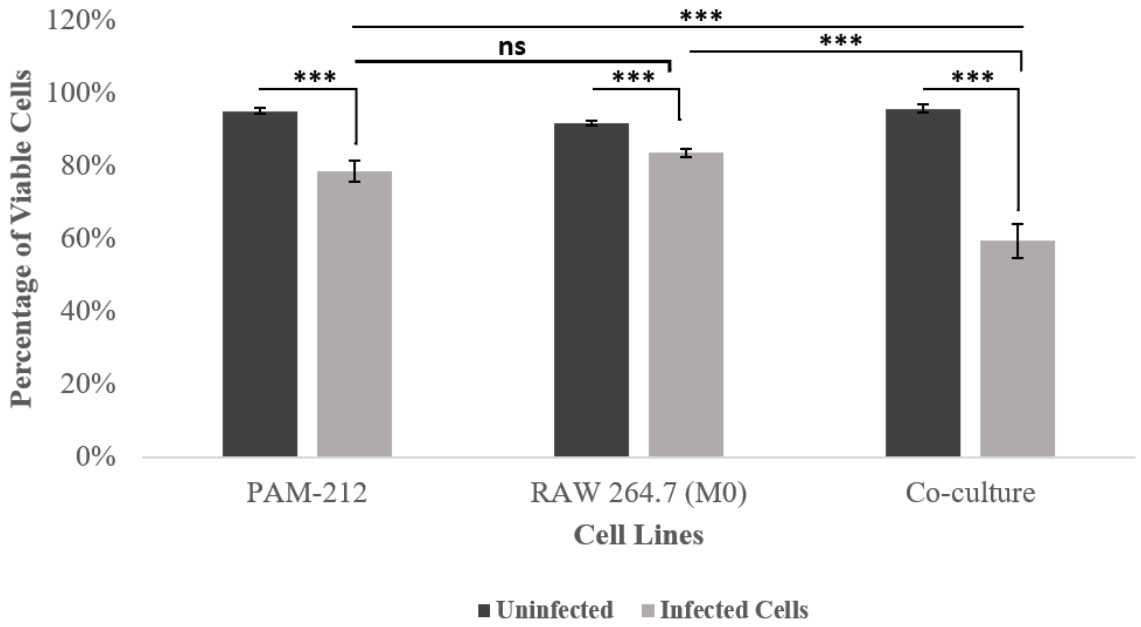


Figure 10: Cell Viability obtained by Trypan Blue Exclusion Test utilizing hemocytometer for (RAW 264.7, PAM-212, and Co-culture) at 48 Hours post-infection with HSV-1.
All monolayers and co-cultured cell lines (PAM-212, RAW 264.7/M0, and Co-culture) exhibited a significant decrease in cell viability at 48 post infection (~16.6%, 8.1%, & 36.3%; P-value = <0.001 P-value = <0.001, P-value = <0.001) respectively were compared to control. However; in comparison between the different of cell lines, there was no significant difference between the keratinocytes and macrophages M0 monolayers infected groups (~5.1 %, P-value = 0.282) while the co-cultured cell lines infected group showed a grate significant decrease in cell viability at 48 hours (~19 %, 24.1%; P-value = <0.001, P-value = <0.001) compared to (PAM-212 & RAW 264.7/M0) respectively. The mean illustrates the percentages of viable cells and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level, ***, $p \leq 0.001$; ns = no significance. (n= 3)

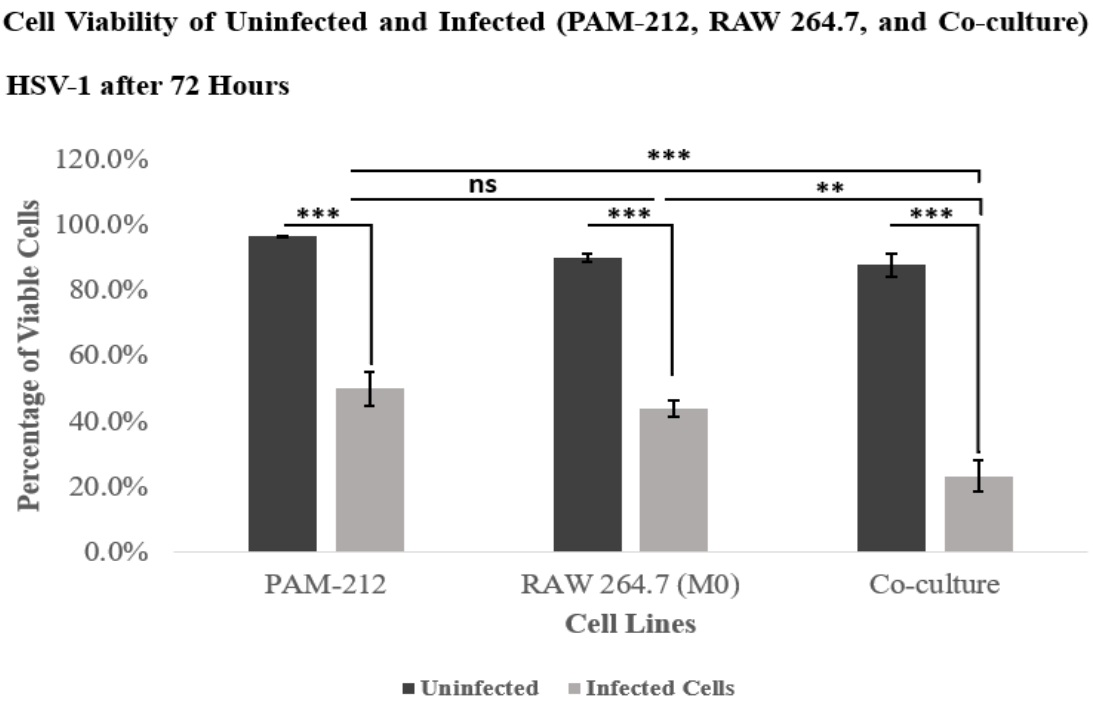


Figure 11: Cell Viability obtained by Trypan Blue Exclusion Test utilizing hemocytometer for (RAW 264.7, PAM-212, and Co-culture) at 72 Hours post-infection with HSV-1.

All monolayers and co-cultured cell lines (PAM-212, RAW 264.7/M0, and Co-culture) exhibited a grate significant decrease in cell viability at 72 post infection (~46.7%, 46.3%, & 64.6%; P-value = <0.001 P-value = <0.001, P-value = <0.001) respectively. However; in comparison between the different of cell lines, there was no significant difference between the keratinocytes and macrophages M0 monolayers infected groups (~6.1%, P-value = 0.323) while the co-cultured cell lines infected group showed a grate significant decrease in cell viability at 72 hours (~26.7 %, 20.6%; P-value = <0.001, P-value = 0.003) compared to (PAM-212 & RAW 264.7) respectively. The mean illustrates the percentages of viable cells and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level, **, $P \leq 0.01$; ***, $p \leq 0.001$; ns, $P > 0.05$ = no significance. (n= 3)

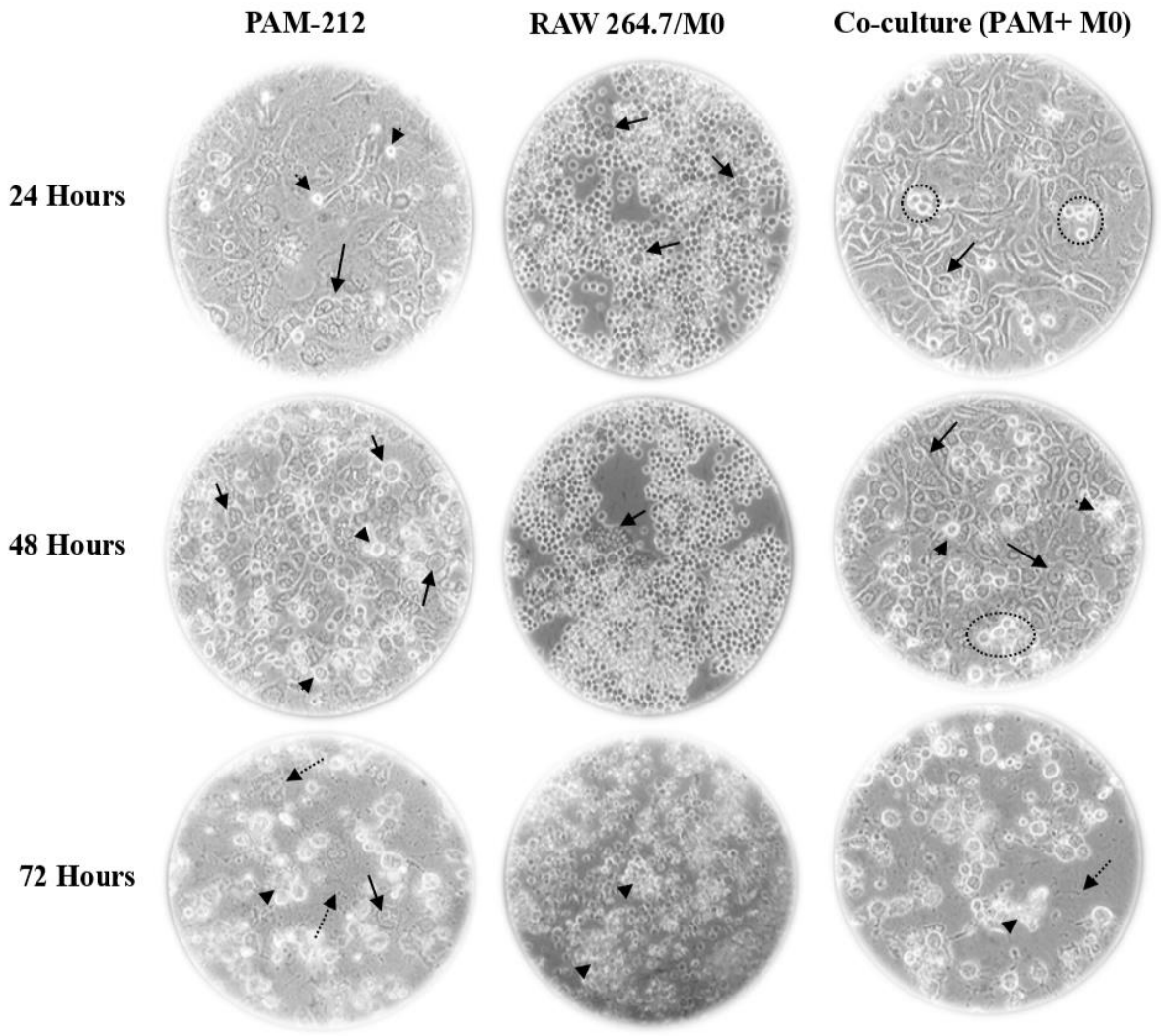


Figure 12: Morphological Changes exhibited by Keratinocytes (PAM-212), Macrophages (RAW 264.7), Co-culture at Time-points of 24, 48, and 72 hours post-infection with HSV-1. The solid arrow exhibits the changes in morphology. The head's arrows show floating cells. The dotted arrows show debris while the dotted circles illustrate the macrophages co-cultured with keratinocytes.

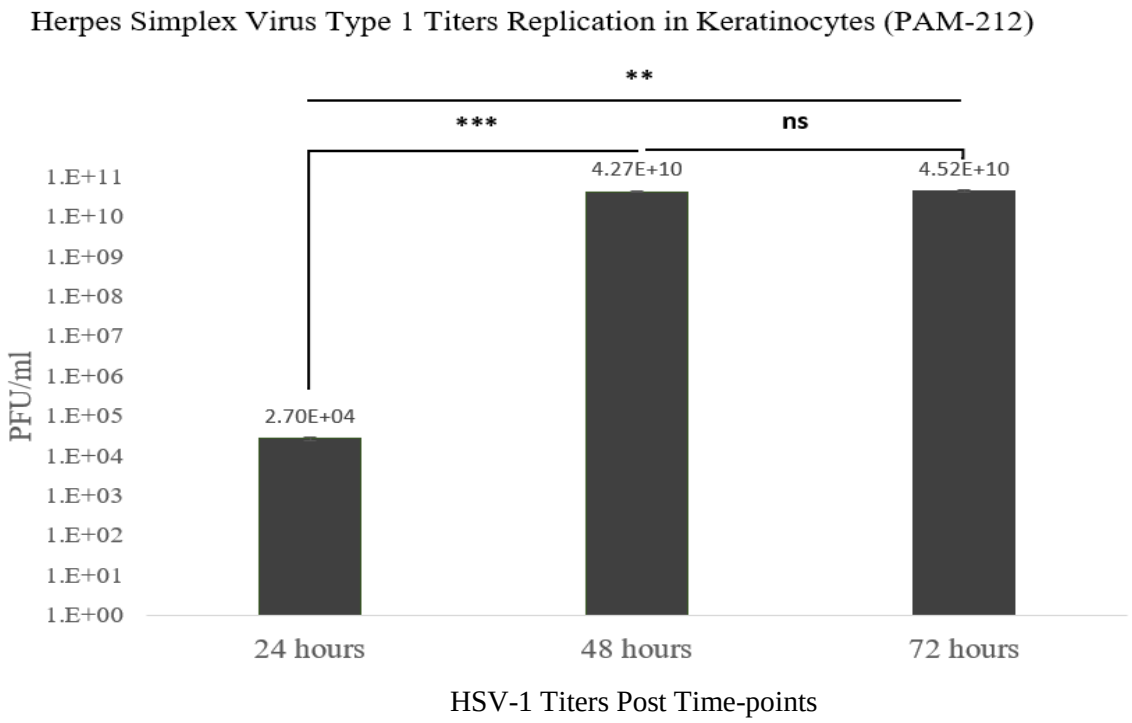


Figure 13: The Plaque Assay for Keratinocytes (PAM-212) Infected with HSV-1 at 24, 48, and 72 Hours. In keratinocytes (PAM-212) at 24 hours, plaques formation was observed from 10^{-1} to 10^{-5}. However, the virus titers were counted at $x10^{-3}$, and the plaque formed per ml (PFU/ml) was 12.6 X 10^{-3} in average. At 48 hours, plaques formation was observed from 10^{-1} to 10^{-10}. The virus titers were counted at $x10^{-8}$, and the plaque formed per ml (PFU/ml) was 214 X 10^{-8} in average. While at 72 hours, plaques formation was observed from 10^{-1} to 10^{-10}. The virus titers were counted at $x10^{-8}$, and the plaque formed per ml (PFU/ml) was 224 X 10^{-8} in average. On the other hand, HSV-1 titers were significantly increased in the keratinocytes from 24 to 48 and 72 hours respectively. In contrast, there was no significant difference between virion replication at 48 and 72 hours. The mean illustrates virus titers and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level, **, $P \leq 0.01$; ***, $p \leq 0.001$; ns, $P > 0.05$ = no significance. (n= 3)

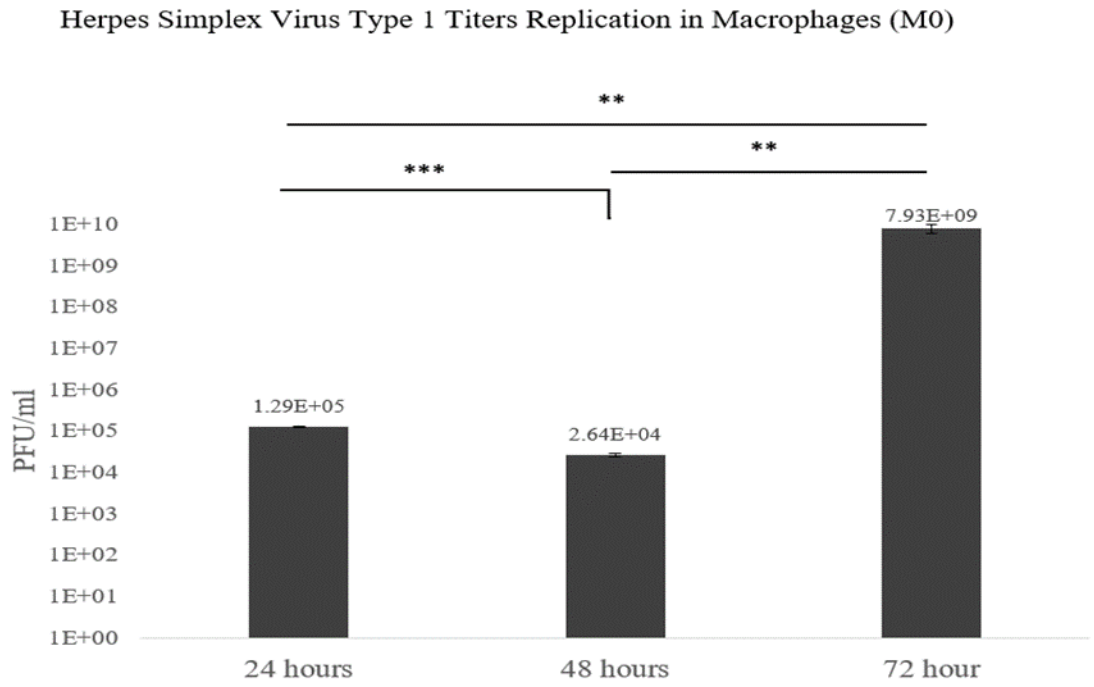


Figure 14: The Plaque Assay for Macrophages (RAW264.7) Infected with HSV-1 at 24, 48, and 72 Hours. In macrophages (RAW 264.7/ M0) at 24 hours, plaques formation was observed from 10^{-1} to 10^{-5}. However, the virus titers were counted at $x10^{-3}$, and the plaque formed per ml (PFU/ml) was 65.6 X 10^{-3} in average. At 48 hours, plaques formation was only observed from 10^{-1} to 10^{-5} and there were no plaques formation beyond the dilution factor $x10^{-5}$ as was demonstrated by six trials. The virus titers were counted at $x10^{-2}$, and the plaque formed per ml (PFU/ml) was 132 X 10^{-2} in average. While at 72 hours, plaques formation was observed from 10^{-1} to 10^{-10}. The virus titers were counted at $x10^{-8}$, and the plaque formed per ml (PFU/ml) was 91 X 10^{-8} in average. Moreover, HSV-1 titers were remarkably decreased in the macrophages (M0) from 24 to 48 hours. However, there was a large significant increase in virion titers at 72 hours compared to 24 and 48 hours. The mean illustrates virus titers and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level, **, $P \leq 0.01$; ***, $p \leq 0.001$. (n= 3)

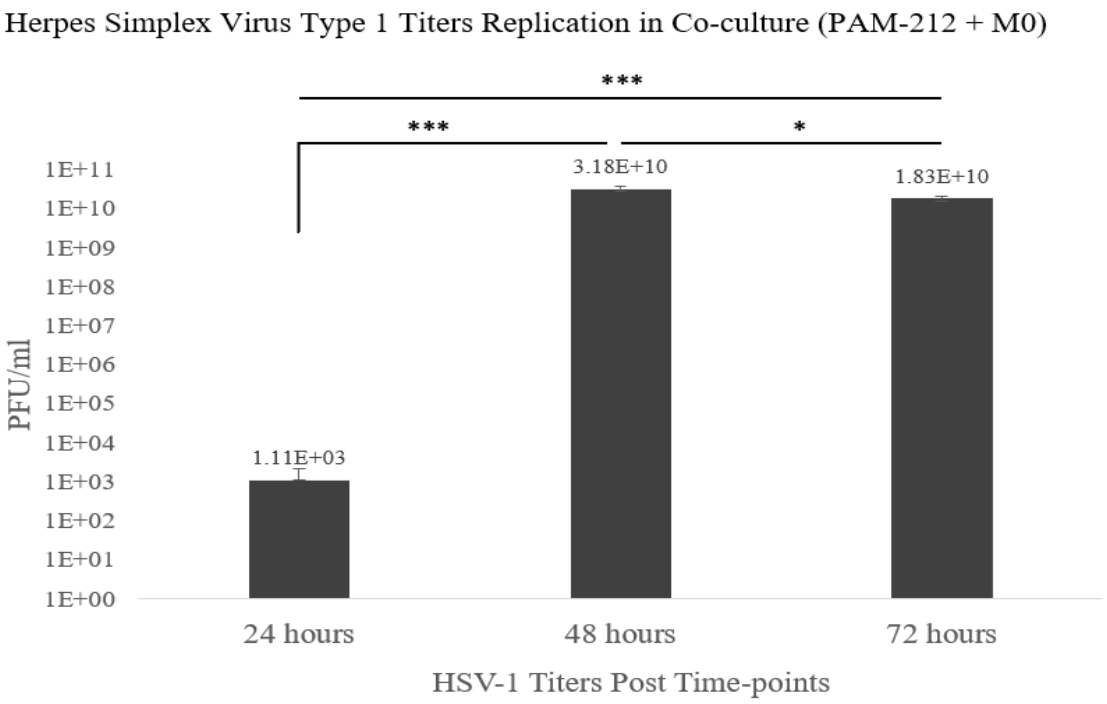


Figure 15: The Plaque Assay for Co-culture (PAM-212 + M0) Infected with HSV-1 at 24, 48, and 72 Hours. In co-culture model (PAM-212 + RAW 264.7/M0) at 24 hours, plaques formation was observed only at 10^{-1} in one plate while other plate did not show any plaques formation beyond the dilution factor $x10^{-1}$ as was demonstrated by four trials. However, the virus titers were counted at $x10^{-1}$, and the plaque formed per ml (PFU/ml) was 55.3 X 10^{-1} in average. At 48 hours, plaques formation was observed from 10^{-1} to 10^{-10}. The virus titers were counted at $x10^{-8}$, and the plaque formed per ml (PFU/ml) was 34.6 X 10^{-8} in average. While at 72 hours, plaques formation was observed from 10^{-1} to 10^{-10}. The virus titers were counted at $x10^{-8}$, and the plaque formed per ml (PFU/ml) was 91.3 X 10^{-8} in average. However, HSV-1 titers were noticeably decreased in the co-culture model at 24 compared to virus titers at 48 and 72 hours respectively. Nevertheless, there was a slightly difference between virion concentrations at 48 and 72 hours. The mean illustrates virus titers and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level, *, $P \leq 0.05$; ***, $p \leq 0.001$. (n= 3)

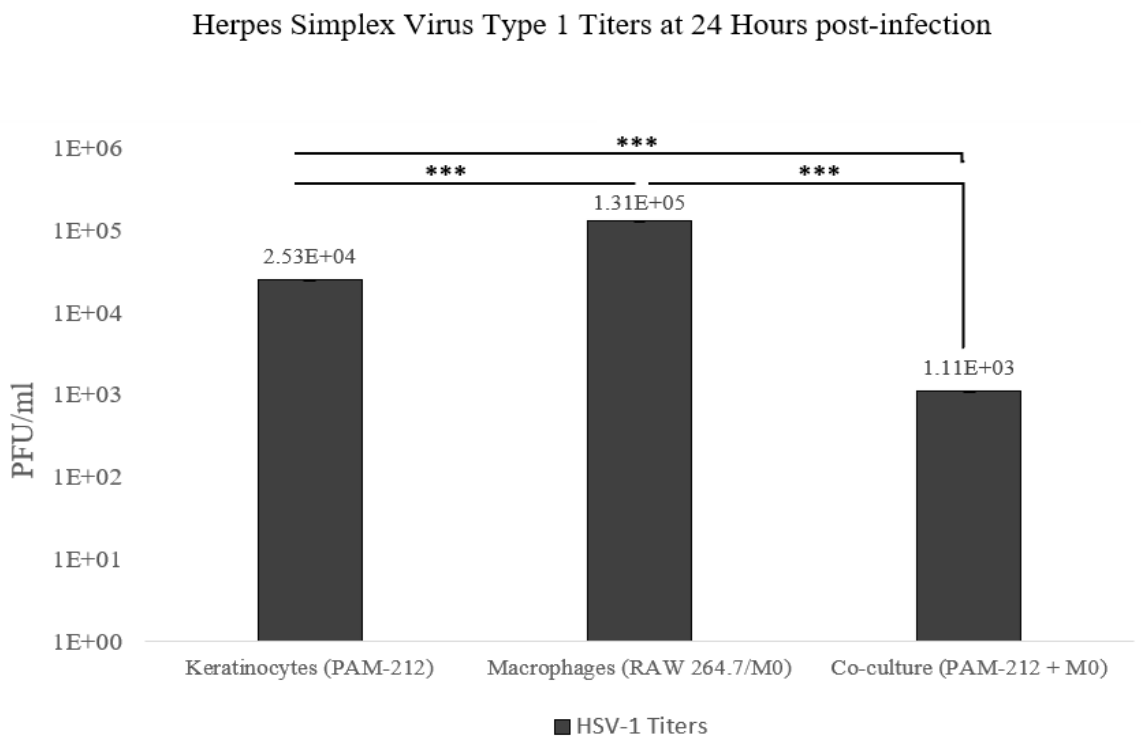


Figure 16: The Plaque Assay compared between all Cell Lines (RAW 264.7, PAM-212, and Co-culture) Infected with HSV-1 after 24 Hours. The keratinocytes (PAM-212) monolayer exhibited less significant in virus concentration compared with macrophages at 24 hours (~5.1fold, P-value = <0.001). While the macrophages (RAW 264.7 /M0) monolayer showed the most significant increase in HSV-1 replication at 24 hours compared to 24 and 72 hours (~5.1 fold, 118 fold, P-value = <0.001, P-value = <0.001) respectively. On the other hand, the co-cultured cell lines (PAM-212 + RAW 264.7/M0) exhibited the lowest significant in virion titers compared to the monolayers infected groups at 24 hours (~22.7 fold, 118 fold, P-value = <0.001, P-value = <0.001) respectively. The mean illustrates virus titers and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level, ***, $p \leq 0.001$. (n= 3)

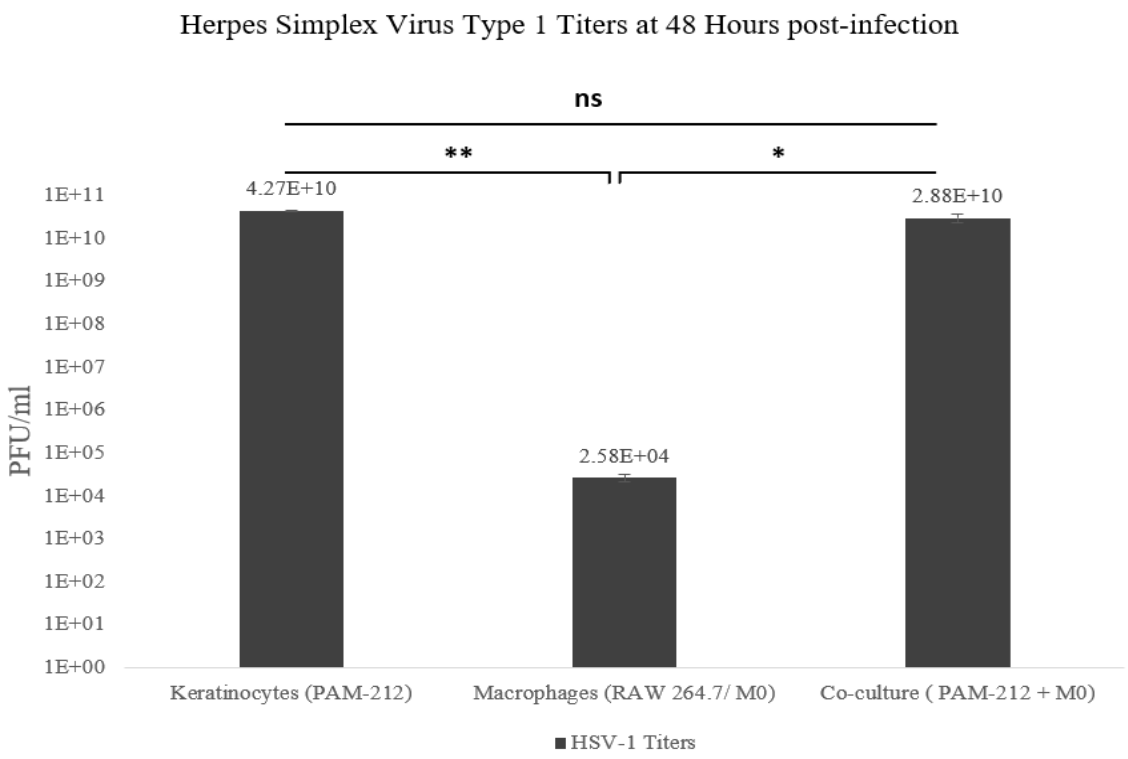


Figure 17: The Plaque Assay compared between all Cell Lines (RAW 264.7, PAM-212, and Co-culture) Infected with HSV-1 after 48 Hours. The keratinocytes (PAM-212) monolayer exhibited no significant in virus concentration compared with co-culture at 48 hours (~1.5 fold, P-value = 0.078). While the macrophages (RAW 264.7 /M0) monolayer showed the lowest significant in HSV-1 replication at 48 hours compared to 24 and 72 hours (~1.65X10^6 fold, 1.11X10^6 fold, P-value = 0.008, P-value = 0.012) respectively. The mean illustrates virus titers and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level, * , $P \leq 0.05$; **, $p \leq 0.01$; ns, $P > 0.05$ = no significance. (n= 3)

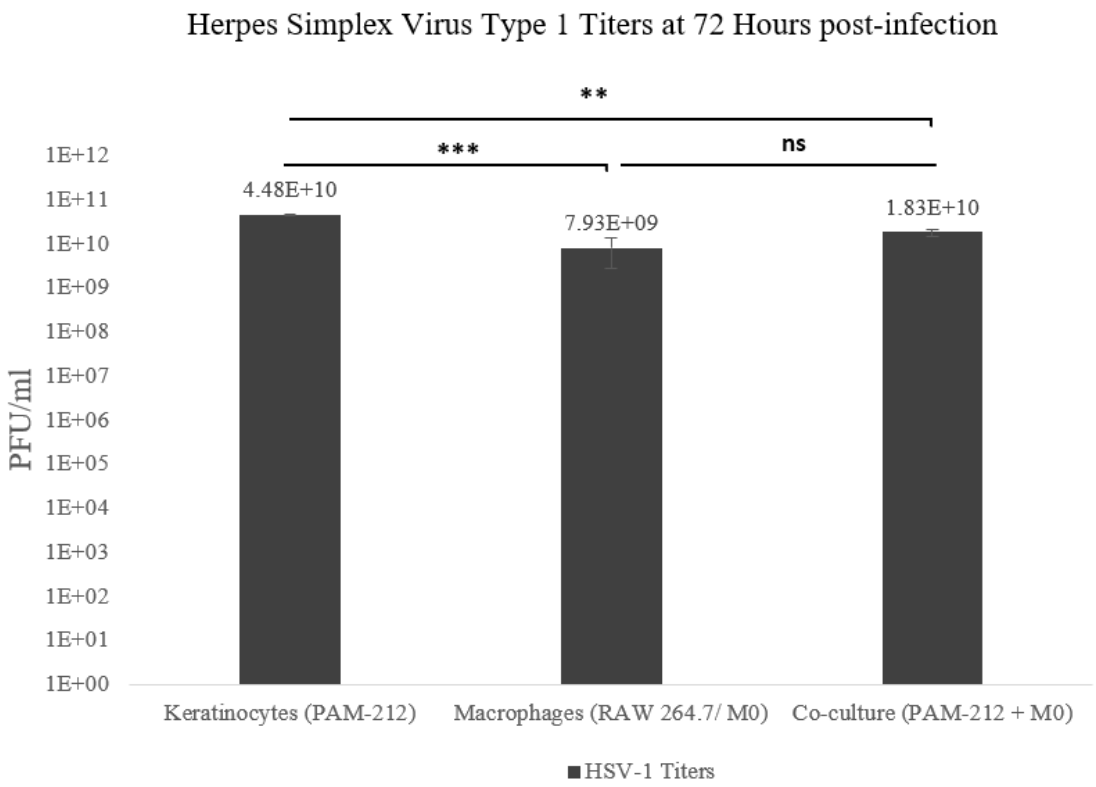


Figure 18: The Plaque Assay compared between all Cell Lines (RAW 264.7, PAM-212, and Co-culture) Infected with HSV-1 after 72 Hours. The keratinocytes (PAM-212) monolayer exhibited a significant increase in virus concentration compared with macrophages (M0) and co-culture at 72 hours (~5.6 fold, 2.44 fold, P-value = <0.001, P-value = <0.001). While the macrophages (RAW 264.7 /M0) monolayer showed the less significant in HSV-1 replication at 72 hours compared to keratinocytes and co-culture (~5.6 fold, 2.3 fold, P-value = <0.001, P-value = 0.052) respectively. The mean illustrates virus titers and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied. Stars (*) indicate the significance level, **, $p \leq 0.01$, ***, $p \leq 0.001$; ns, $P > 0.05$ = no significance. (n= 3)

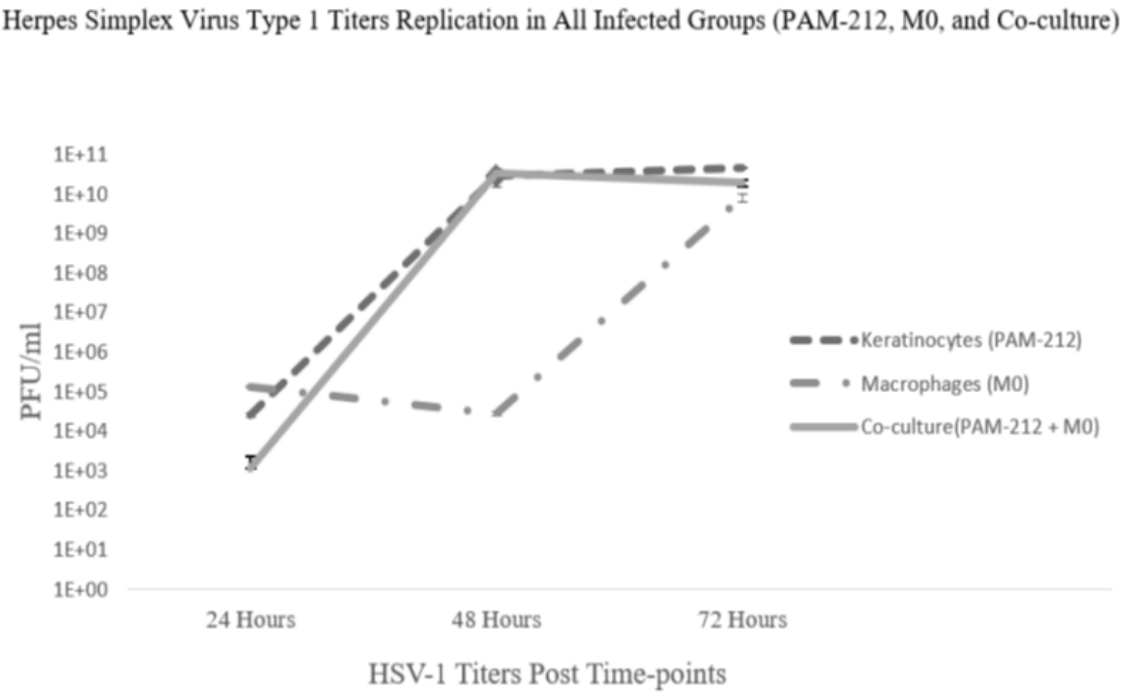
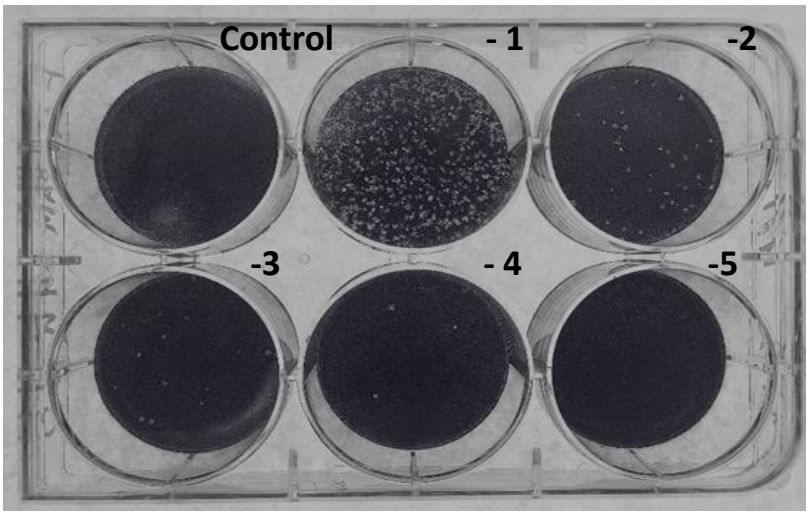
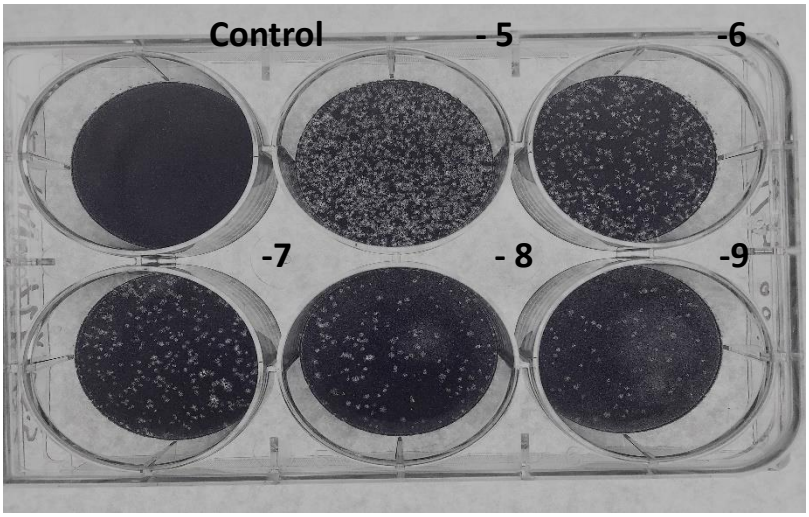


Figure 19: This Line Graph illustrates and Summarizes the Plaque Assay compared between all Cell Lines (RAW 264.7, PAM-212, and Co-culture) Infected with HSV-1 after 24, 48, and 72 Hours. The dark dotted line represents the keratinocytes (PAM-212), the gray dotted line represents the macrophages (M0), and the solid line represents the co-culture. The mean illustrates virus titers and error bars represent standard error ±. The statistics were obtained utilizing Sigma-Plot 12.0, One-way ANOVA was applied.

This plate shows plaques formation for PAM-212 infected with HSV-1 at MOI 0.1 for 24 hours.



This plate shows plaques formation for PAM-212 infected with HSV-1 at MOI 0.1 for 48 hours.



This plate shows plaques formation for PAM-212 infected with HSV-1 at MOI 0.1 for 72 hours.

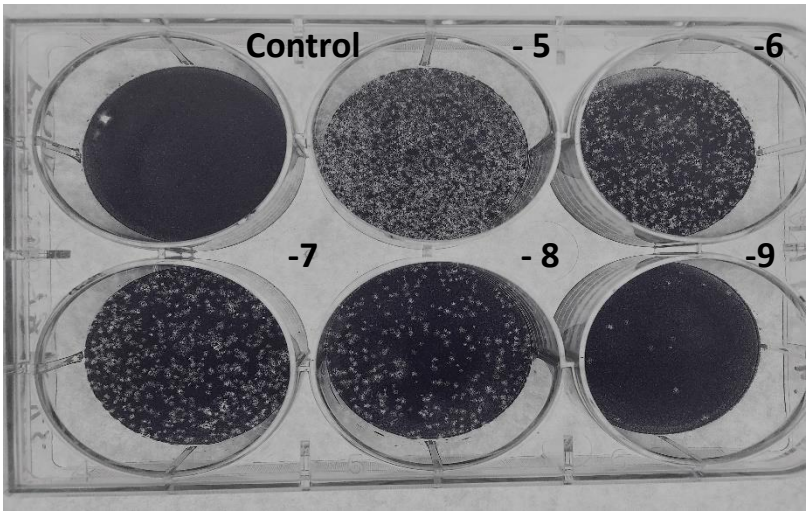
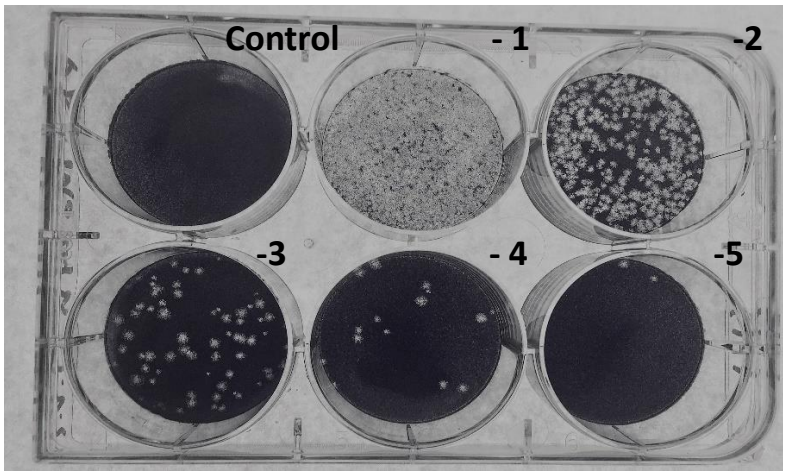
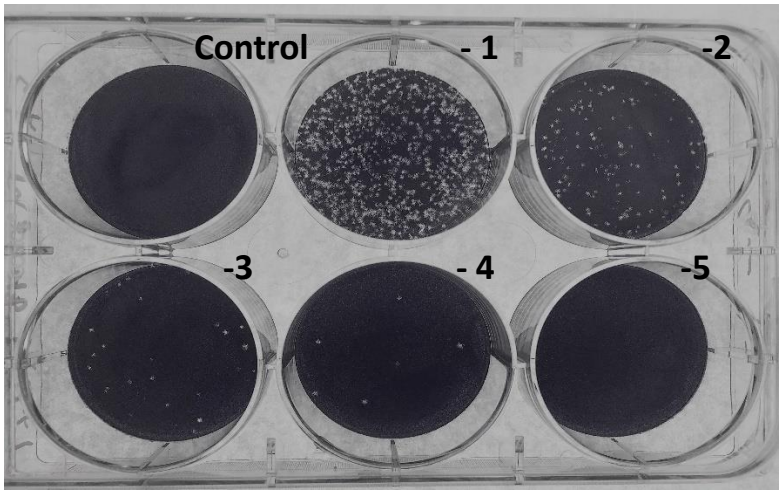


Figure 20: Plaque Formation for Keratinocytes (PAM-212) after 24, 48, 72 hours

This plate shows plaques formation for RAW 264.7 infected with HSV-1 at MOI 0.1 for 24 hours.



This plate shows plaques formation for RAW 264.7 infected with HSV-1 at MOI 0.1 for 48 hours.



This plate shows plaques formation for RAW 264.7 infected with HSV-1 at MOI 0.1 for 72 hours.

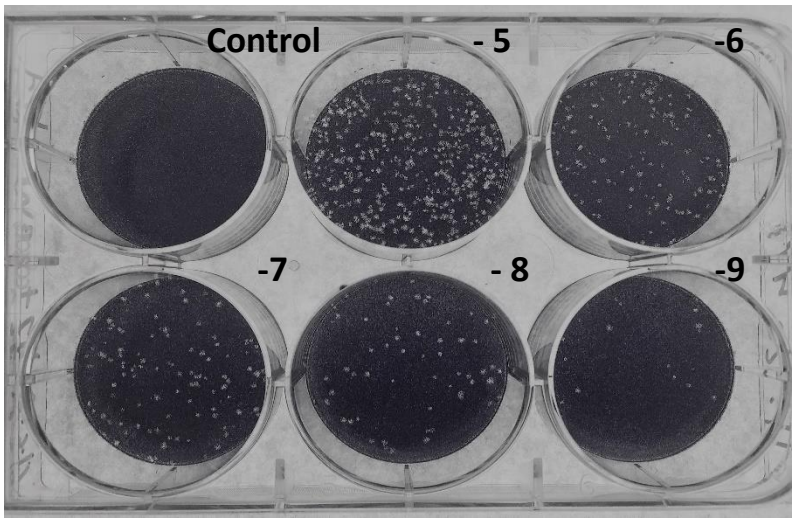
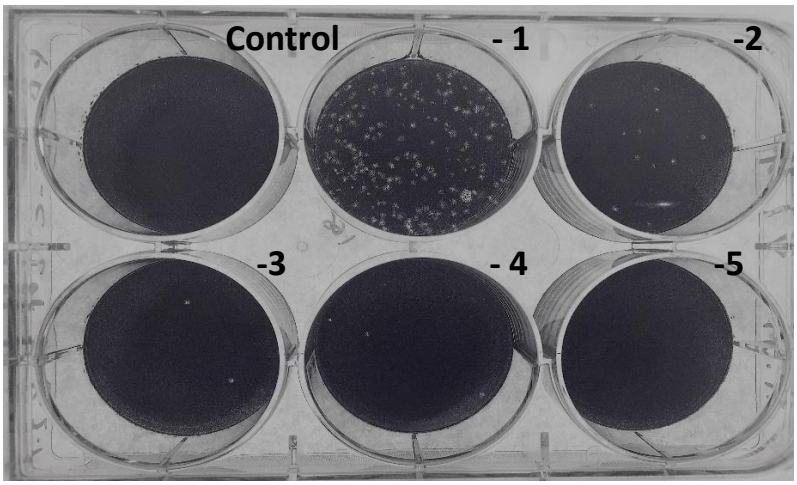
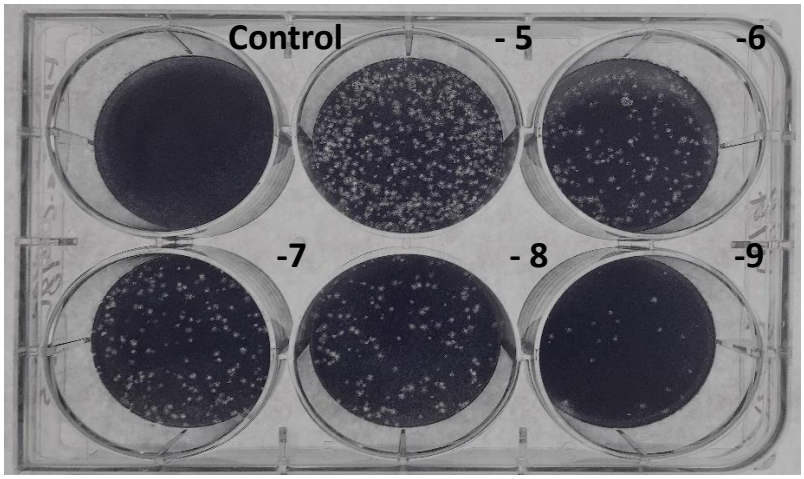


Figure 21: Plaque Formation for Macrophages (RAW 264.7) after 24, 48, 72 hours

This plate shows plaques formation for co-culture PAM-212 + RAW 264.7 infected with HSV-1 at MOI 0.1 for 24 hours.



This plate shows plaques formation for co-culture PAM-212 + RAW 264.7 infected with HSV-1 at MOI 0.1 for 24 hours.



This plate shows plaques formation for co-culture PAM-212 + RAW 264.7 infected with HSV-1 at MOI 0.1 for 24 hours.

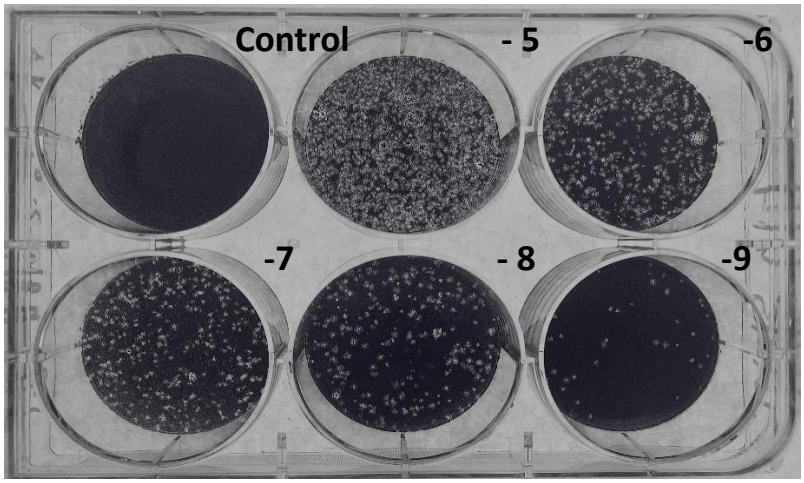


Figure 22: Plaque Formation for Co-culture (PAM-212 & RAW 264.7) after 24, 48, 72 hours

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